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The Effect of Estrogen in a Murine Model of Peritoneal Adhesion Formation

Michelle Rene Frazier-Jessen
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

THE EFFECT OF ESTROGEN IN A MURINE MODEL OF PERITONEAL ADHESION FORMATION

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MICHELLE RENE FRAZIER-JESSEN

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LIST OF ABBREVIATIONS

CAPD	continuous ambulatory peritoneal dialysis
E_2	17-β-estradiol
ER	estrogen receptor
ERE	estrogen response element
IL-1	interleukin-1
IL-2	interleukin-2
IL-8	interleukin-8
LDL	low density lipoprotein
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
mRNA	messenger ribonucleic acid
OVX	ovariectomized
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PEC	peritoneal exudate cells
PKC	protein kinase c
PMA	phorbol myristate acetate
PMN	polymorphonuclear leukocyte
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha

CHAPTER 1

INTRODUCTION

Inflammation is the process by which proteins and cells accumulate at a site of tissue damage or infection (for a review, see Kovacs and Frazier-Jessen 1994). The activation of the cells at these sites leads to the removal of necrotic tissue and either regeneration of the normal tissue or replacement with scar tissue. The inflammatory process is designed to provide a rapid mechanism by which the host can respond to the invasion of foreign materials and return to a homeostatic equilibrium. Excessive or inadequate activation of the system can have serious effects, as can the failure of inactivation mechanisms. In these instances, the end result is wounds that never heal, wounds that heal poorly, or wounds that heal too much (Figure 1). Wounds that never heal include most neoplasms. Wounds that heal poorly are represented by cutaneous ulcers, a common complication in diabetics and other individuals with circulatory problems. Wounds that heal too much result in an overproduction of connective tissue that severely limits and often destroys the functionality of the organ tested. In the skin, this results in severe scarring, such as keloid formation. In other organs such as lung, liver, and peritoneum, the end result is fibrosis and/or adhesion formation. Fibrosis and adhesion formation in the peritoneal cavity can lead to formation of bowel strictures (Fabri and Rosemurgy 1991; Krebs and Goplerud 1987), a life-threatening consequence that requires immediate medical intervention. Furthermore, adhesion formation is an important pathological consequence of endometriosis, one of

PATHOBIOLOGY OF WOUND REPAIR

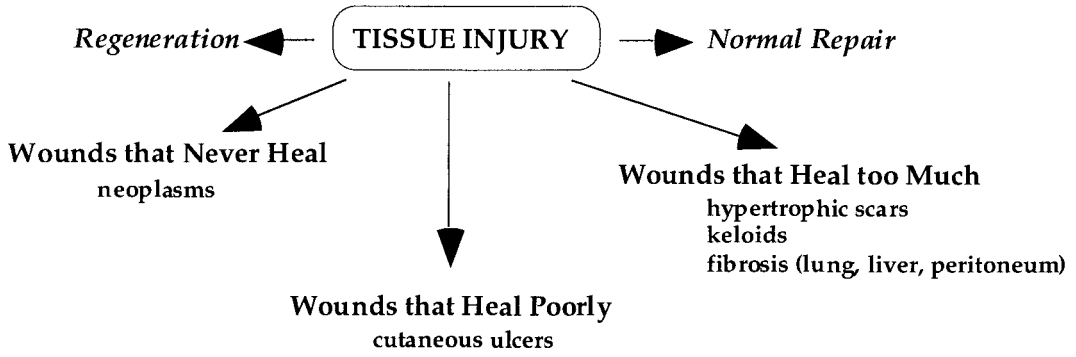

Figure 1. The Pathobiology of Wound Repair.

the major causes of infertility (Haney 1993). Therefore, a more accurate understanding of the regulation of the inflammatory events would aid in prevention of these pathologic consequences or the development of effective therapeutic agents.

Studies have shown that one immune cell population essential for resolution of the wound is the macrophage (Leibovich and Ross 1975). The wound macrophage is not only responsible for the phagocytosis of foreign material/debris, but probably most important, the secretion of a wide assortment of mediators known as cytokines that aid in cellular recruitment, stimulation of new matrix production and proliferation of fibroblasts (Kovacs 1991). While the fibroblast itself is critical in the production of new matrix components, it is essentially under the control of these macrophage-derived mediators. One cytokine that has been shown to significantly effect the outcome of wound healing is monocyte chemotactic protein-1 (MCP-1). As its

name implies, this cytokine is a potent chemotactic and activating factor for monocytes and macrophages (Rollins, Walz, and Baggiolini 1991; Yoshimura and Leonard 1990; Yoshizuka et al. 1989; Zachariae et al. 1990).

Most studies involving the immune response to inflammation to date have utilized male animals, and those involving females have ignored the cyclic fluctuation of sex steroid hormones associated with the estrus cycle (Dresser 1992). Recent studies suggest that endogenous female hormones may affect a number of processes involved in the immune response. Since hormones are likely to play an integrative role in the development of the response to injury, endocrine disorders can impair host defenses to infection. It is, therefore, relevant to understand how hormones regulate the initiation and subsequent course of the inflammatory reaction. This is especially important when considering drug therapy, as it is necessary to have an understanding of the potential modulatory influences of these hormones.

Hypothesis

Estrogen modulates connective tissue deposition in the peritoneal cavity in response to talc-induced injury. This modulation of connective tissue deposition occurs, in part, through the decreased migration of macrophages into the inflammatory site via inhibition of the macrophage chemoattractant protein, JE.

Specific Aims

Aim 1: To develop a murine model of peritoneal adhesion formation in the peritoneal cavity and a quantitative means for assessing it.

Rationale: Peritoneal fibrosis is a common clinical problem whose treatment is currently limited to surgical intervention. Current assessment techniques are limited to ranking of adhesions following visual inspection. A model system with a means of more accurate assessment would allow for analysis of therapeutics in the treatment of this complication.

Aim 2: To examine the effect of estrogen on connective tissue deposition in a murine model of peritoneal adhesion formation.

Rationale: Estrogen plays a role in connective tissue remodeling in reproductive organs and it has also been shown to have effects on the inflammatory response, the event preceding connective tissue deposition. It is, therefore, conceivable that estrogen can modulate connective tissue remodeling in response to an inflammatory event.

Aim 3: To examine the effect of estrogen on gene expression of JE, a macrophage chemotactic factor, in primary culture macrophages and macrophage cell lines.

Rationale: Macrophage recruitment, via chemotactic factors such as JE, is critical to the resolution of an inflammatory stimulus. As estrogen has previously been shown to alter production of other macrophage-derived cytokines, it is conceivable that estrogen could alter

JE gene expression and thus modulate the ultimate outcome of inflammation.

CHAPTER 2

REVIEW OF THE RELATED LITERATURE

The Inflammatory Response

The inflammatory response can be divided into a series of overlapping stages: acute vascular, acute cellular, chronic cellular, and resolution (Kovacs and DiPietro 1994). This response is triggered by trauma, infection, tissue necrosis, or immune reaction, which leads to the dilation of blood vessels causing the leakage of fluid from precapillary arterioles. Acute changes in blood flow pattern lead to margination of neutrophils along the vascular surface of the endothelial cells, followed by the directed migration of neutrophils through the endothelial cell monolayer into the connective tissue below. Depending on the degree of infection or tissue damage, this process may be sufficient to remove debris and to restore normal tissue structure. However, a chronic inflammatory infiltrate of macrophages and lymphocytes is usually necessary to remove bacteria and necrotic tissue. When the damage is more extensive, normal tissue is replaced in the resolution phase by scar tissue, a result of the proliferation of fibroblasts and the depositing of connective tissue proteins.

Aberrant Wound Healing

The return to normal tissue structure and function following a local inflammatory event depends o the degree of damage to the tissue and the

ability of cells within the tissue to regenerate. In the lung, for example, after the removal of the epithelium, a "competition" arises between reepithelialization and fibrosis (Hascheck and Witschi 1979; Haslett and Henson 1988; Terzaghi, Nettesheim, and Williams 1978). In the absence of inhibitory signals, the aberrant production of these mediators sustains the connective tissue architecture.

The overproduction of connective tissue can permanently damage or severely limit the functionality of the organ involved. Examples of overhealing in the skin include hypertrophic scarring, keloid formation, and third degree burns. In other organs, such as the lung, liver and peritoneal cavity, this results in fibrosis and/or adhesion formation (see Figure 1, Chapter 1).

The Peritoneum and Adhesion Formation

The peritoneal cavity is the largest cavity in the body and the lining of its surface area equals that of the skin. The lining of the peritoneal cavity, called the peritoneum, is composed of a single layer of mesothelium that rests on a thin layer of fibroelastic connective tissue (Figure 2, Part A). In the absence of pathology, the peritoneal cavity contains a minimal amount of peritoneal fluid (Padawer 1973).

The irritation and inflammation of the peritoneum is known as peritonitis and is almost always preceded by some kind of intervention into the peritoneal cavity. During this inflammatory state, there is an outpouring of exudate which contains fibrinogen and may cause the intraperitoneal tissues to adhere to their neighbors (Myllarniemi et al. 1966). While the majority of these fibrinous adhesions are transient, some become fibrous by the ingrowth of fibroblasts and blood vessels (Jackson 1958).

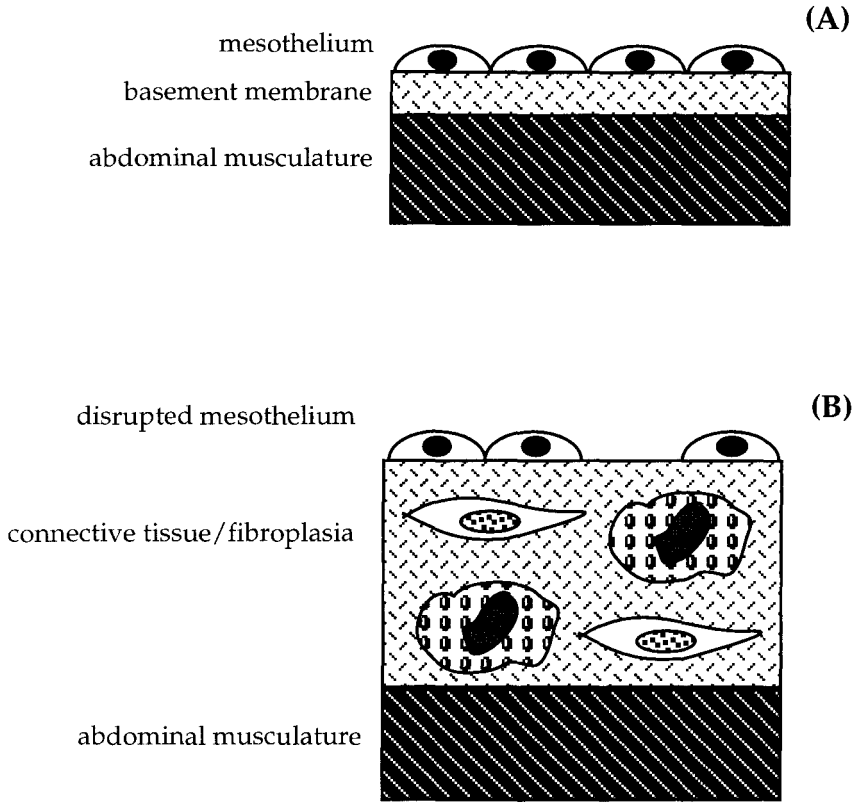

Figure 2. The Peritoneal Membrane (A) under normal, noninflammatory conditions; (B) after injury (fibrosis) with increased connective tissue deposition along with migration of fibroblasts and macrophages into the submesothelial space.

Although the process of healing follows similar patterns in all wounds, peritoneal healing is different from skin wound healing in that healing of the mesothelium takes place throughout the whole surface and even extended defects of the peritoneum are completely resolved within 7-8 days (Raftery 1973a; Raftery 1973b; Raftery 1973c). In contrast, epithelialization of the skin starts from the wound edges and is a slower, more prolonged process.

Within a short time period post-injury, a fibrin network appears in the abdominal cavity (Myllarniemi et al. 1966). The local coagulative and

fibrinolytic properties of the peritoneum largely determine the final state of adhesion formation (Gervin, Puckett, and Silver 1973; Porter, Ball, and Silver 1971). If fibrinolysis is adequate or the damage is moderate, adhesions may disappear (Buckman et al. 1976). However, if the injury is extensive or the irritating substance cannot be cleared, extensive adhesions can appear within 24 hours. Fibroblasts then proliferate into the fibrin and turn it into fibrous adhesions (Myllarniemi et al. 1966) (Figure 2, Part B). Fully developed adhesions may be observed from 10 days to 2 weeks (Welte, Albinus, and Dominick 1973).

An important factor in adhesion formation is the chemotactic, phagocytic and fibrogenic functions of PMNs and macrophages (Curran and Clark 1964). Macrophages are especially critical in the final resolution of tissue debris and completion of healing, a process that ends in the formation of connective tissue matrix and mesothelial syncytium (Bryant et al. 1988).

Causes of Peritoneal Adhesions

The production of scar tissue within the peritoneal cavity can result from surgery, infection, introduction of foreign materials, or intraperitoneal immunotherapy regimens to treat abdominal tumors. It is also a complication for renal dialysis patients undergoing continuous ambulatory peritoneal dialysis (CAPD) and for women who suffer from endometriosis.

Surgical Intervention

Surgical procedures in the abdominal cavity can often result in the formation of peritoneal adhesions. These adhesions can result from tissue ischemia, excessive bleeding, and thermal injury during the surgical procedure.

As a result of tissue injury, post-surgical resident peritoneal macrophages secrete factors that either suppress or enhance the proliferation peritoneal tissue repair cells, in addition to modulating the morphology and secretion of extracellular matrices by these cells (Rodgers and DiZerega 1992).

Furthermore, various kinds of irritants which are only slowly absorbed such as glove powders, surgical materials, materials applied intraperitoneally for investigative purposes or for the treatment of peritonitis can induce granulation tissue and adhesions. If the inflammatory state turns chronic, the proliferation of fibroblasts and connective tissue formation continues and after several months appears as fibrosis.

Talc, a form of silica, is used during the construction of synthetic gloves. This substance, which is poorly, if at all, cleared by the immune system, induces a strong granulomatous foreign body reaction (Eismann, Seelig, and Womack 1947). Many cases of peritoneal adhesion formation are the result of talc contamination secondary to abdominal surgery (Henderson et al. 1978; Kus et al. 1979).

Intraperitoneal Immunotherapy

The use of intraperitoneal immunotherapy for the treatment of tumors localized in the peritoneal cavity has gained widespread popularity in recent years. In these instances, intraperitoneal (i.p.) therapy affords a higher concentration of infused biological response modifiers than does intravenous administration. One method of i.p. immunotherapy involves the culture of autologous peripheral blood leukocytes with interleukin-2 (IL-2) to generate lymphokine-activated killer (LAK) cells. LAK cells are extremely effective at killing tumor cells in vitro (Grimm et al. 1982). The i.p. infusion of LAK cells

and additional IL-2 has had promising preliminary results in combating ovarian tumors in vivo (Urba et al. 1989). One of the complications arising form LAK cell/IL-2 therapy is the development of peritoneal fibrosis (Lotze, Custer, and Rosenberg 1986; Steis et al. 1990; Urba et al. 1989). Dense adhesions form within the peritoneal cavity, initially around the catheter site. These adhesions prevent the LAK cells from reaching their tumor targets. Patients must then be removed from therapy, and in some cases, undergo surgery to lyse adhesions. The development of these adhesions has not been clearly elucidated, but could be due to the production of fibrogenic mediators by the LAK cells (Kovacs et al. 1989; Kovacs et al. 1990) or by IL-2-stimulated resident peritoneal cells (Kovacs and Neuman 1991; Kovacs, Stedum, and Neuman 1994) in response to the treatment, the tumor cells themselves, either alone or in combination. It would, therefore, be of interest to further investigate each of these areas.

Continuous Ambulatory Peritoneal Dialysis and Peritoneal Adhesions

Continuous ambulatory peritoneal dialysis (CAPD) is an important and effective method of treatment for end-stage renal disease. However, this therapy is characterized by a nearly 50% chance of patients developing recurrent peritonitis (Golper and Hartstein 1986; Schneble et al. 1992). CAPD patients present with peritoneal alterations characterized by a diffuse thickening of the peritoneal membrane due to an increased amount of connective tissue (Daugirdas et al. 1986; Gandhi et al. 1980). This fibrotic response remains one of the major causes of "dropout" of CAPD patients, due to a decrease in ultrafiltration capacity by the peritoneal membrane (Carozzi 1990; Nolph et al. 1984). Peritoneal macrophages play an important role in the

pathology observed in these patients. This macrophage population is characterized by a highly activated, immature phenotype, such as those seen in chronic inflammatory diseases (Betjes et al. 1993). During episodes of peritonitis, these macrophages secrete high levels of cytokines such as IL-1, TNF-α (Douvdevani et al. 1993; Fieren, Bemd, and Bonta 1990; Fieren et al. 1991), IL-6, and IL-8 (Lin, Lin, and Huang 1993). The presence of such high levels of inflammatory cytokines can result in a chronic inflammatory state and thus lead to the fibrotic response observed in the peritoneal cavity of these patients.

Endometriosis and Peritoneal Adhesions

Endometriosis occurs when normal endometrial tissue grows in extra-uterine locations, such as the peritoneal cavity. This ectopic endometrial tissue may eventually lead to the formation of scar tissue or peritoneal adhesions. Besides causing extreme pain, endometriosis is though to be one of the major causes of infertility. Further complications include bowel obstruction, which must be treated surgically (Krebs and Goplerud 1987).

Some of the modifications seen within the pelvic cavity in patients with endometriosis include an increase in the production of cytokines by infiltrating leukocytes. These cytokines include IL-1 (Fakih et al. 1987), IL-6 (Buyalos et al. 1992), TNF-α (Eisermann et al. 1988; Halme 1989), and PDGF (Halme 1988). In addition, peritoneal fluid from endometriosis patients has higher chemotactic activity for macrophages and monocytes than peritoneal fluid from normal patients, although the identity of the chemotactic factor has not yet been deduced (Leiva et al. 1993).

The source of increased cytokines in the peritoneal cavity of endometriosis patients could be from ectopic endometrium. This tissue has the potential to elaborate a wide variety of cytokines, since the endometrium is a site of active cytokine production (Tabibzadeh 1991). In addition, peritoneal macrophages, which secrete all of the above-mentioned mediators, are present in very high numbers in the peritoneal fluid of endometriosis patients (Badaway et al. 1984; Halme et al. 1983). Not surprisingly, the number of macrophages present in these patients directly corresponds with the severity of the disease (Olive, Weinberg, and Haney 1985).

The cyclic variations in hormone concentrations are responsible for most of the pathological modifications observed in endometriosis patients. Other investigators have shown that cytokine levels and cell populations in the uterus fluctuate during the estrus cycle and that sex hormones regulate these changes (De, Sanford, and Wood 1993; De, Sanford, and Wood 1992; De and Wood 1990; Olive, Weinberg, and Haney 1985). Furthermore, an increase in macrophage and PMN chemotactic activity is observed in the peritoneal fluid of endometriosis patients (Leiva et al. 1993). This chemotactic activity is decreased below that of normal patients in endometriosis patients receiving hormonal treatment. All of these observations suggest that the development and progression of endometriosis by inflammatory cells may be hormonally regulated.

Inflammation and Wound Healing

As discussed above, it is the inflammatory cells present within the damaged tissue site that are responsible for repair and resolution. Of the cell populations present, the macrophage and the fibroblast play direct roles in the

synthesis and remodeling of connective tissue elements necessary to return the damaged tissue to a functional state.

Macrophages

Monocytes constitute 3-8% of the circulating leukocyte population and mature into connective tissue macrophages on migration out of the circulatory system into specific extravascular compartments. After derivation from precursors in the bone marrow, monocytes remain in the circulation for 1-2 days before they differentiate into macrophages.

Macrophages are long-lived, highly motile phagocytic cells with large numbers of lysosomes, endocytotic vacuoles, and intracellular vesicles containing indigestible material. Macrophages ingest extracellular material by phagocytosis and pinocytosis. These internalized components are digested by lysosomal enzymes to clear them from the extracellular environment. On activation, the number of lysosomes per cell increases, as does the amount of hydrolytic enzyme per lysosome, to facilitate the digestion of material taken up by phagocytosis. Under certain conditions, such as the invasion of foreign material, macrophages can form multinucleated giant cells resulting from the fusion of several macrophages. These giant cells can remain in the tissue for extended periods of time and are thought to act as barricades that surround foreign material, thus separating it from normal tissue.

In culture, as well as in vivo, macrophages adhere to surfaces of cells and to extracellular matrix elements, and are able to migrate. In addition to making directed and random movements as cellular entities, macrophages can extend pseudopods in an ameboid fashion to reach out and explore their environment.

Tissue macrophages were initially named for the specific tissues in which they were identified, for example, alveolar or pulmonary macrophages of the lung. Although the macrophages from different tissues share certain "generic" characteristics, some characteristics are tissue specific. For example, because of high concentration of oxygen in the lung, alveolar macrophages have a higher capacity to produce reactive oxygen metabolites than do macrophages from other tissues.

In addition to being migratory and phagocytic, macrophages are also highly secretory, producing a variety of mediators that range from cytokines and prostaglandins to connective tissue elements (for a review, see Nathan 1987). These factors include enzymes (Werb and Gordon 1975a; Werb and Gordon 1975b) and cytokines.

Over the past two decades, activated macrophages have been shown to produce several mediators which play direct or indirect roles in inflammatory and fibrotic processes. These cytokines, which will be referred to as proinflammatory and fibrogenic, include IL-1 (Schmidt et al. 1982), IL-8 (Rot 1993), JE/MCP-1 (Rollins, Walz, and Baggiolini 1991), TNF-α (Beutler et al. 1985), PDGF (Shimakado et al. 1985), FGF (Baird, Morm'ede, and B:ohlen 1985), and TGF-β (Assoian et al. 1987) are discussed later in the text.

Fibroblasts

Fibroblasts are mesenchymal cells present in most organs which play a crucial role in repair processes. Fibroblasts are the most abundant of connective tissue cell types. Although they may be dormant for prolonged periods of time, these cells are capable of returning to the cell cycle on stimulation by appropriate environmental cues. In response to injury, resident fibroblasts in

the surrounding tissue proliferate and then migrate into the wound site (Clark 1993). Once within the wound, they produce high levels of collagen, as well as a variety of other extracellular matrix molecules (Kleinman, Klebe, and Martin 1981), and deposit these molecules into the local milieu. Overproduction of collagen by fibroblasts is felt to be the result of high levels or continued production of cytokines and other inflammatory mediators (Kovacs 1991). Macrophages are known to produce cytokines that modulate growth and collagen synthesis by fibroblasts. PDGF, for example, is one such macrophage-derived factor which induces resident fibroblasts to proliferate (Ross and Raines 1990). In addition, fibroblasts are intimately involved in the feedback mechanisms during the inflammatory response as they also secrete inflammatory cytokines (Cochran, Reffel, and Stiles 1983; Elias, Reynolds, and Lentz 1990; Elias et al. 1989).

Cytokines

Cytokines are soluble protein mediators secreted by cells which regulate immunological and inflammatory events. Generally, cytokines are low molecular weight secretory proteins that are produced transiently and locally. These mediators are extremely potent, working at picomolar concentrations and interacting with high affinity cell surface receptors that are specific for each cytokine or cytokine family. Studies have shown that a number of cytokines can induce fibroblast proliferation and connective tissue deposition (see Kovacs 1991, for a review). These cytokines include TNF-α, IL-1, IL-8, JE/MCP-1, PDGF and TGF-β, all of which can have an important role in determining the outcome of an inflammatory response by their ability to recruit and activate local macrophage populations.

Proinflammatory Cytokines

Proinflammatory, or early inflammatory cytokines are thought to initiate a cascade of events which can lead to fibrosis. A subset of these mediators has been reported to be present in the abdominal cavity. The presence of pelvic pathology, ranging from endometriosis to ovarian cancer, showed a positive correlation with elevatedproduction of cytokines (Buyalos et al. 1992; Halme et al. 1988) and fibronectin (Kauma et al. 1988) by peritoneal lavage cells. Furthermore, elevated levels of fibrogenic cytokines are present in peritoneal fluid from patients given intraperitoneal infusion of lymphokine-activated killer cells and interleukin-2 (IL-2) to combat ovarian tumors (Kovacs and Urba, unpublished observation), in mice given intraperitoneal IL-2 (Frazier-Jessen and Kovacs, unpublished observation) or murine peritoneal macrophages treated with IL-2 in vitro (Kovacs, Van Stedum, and Neuman 1994). Of these cytokines, IL-1, TNF-α and JE/MCP-1 are discussed below.

Interleukin-1

Interleukin-1 (IL-1) was first described in the 1940s as endogenous pyrogen, a substance in acute exudate fluid, which, when administered in vivo, induced fever (reviewed in (Dinarello 1984)). Since then, a multitude of biological actions have been attributed to this cytokine (Oppenheim et al. 1986). IL-1 is felt to be the body's key mediator of acute responses to microbial invasion, immunological reaction, inflammation and tissue injury. The biological effects of IL-1 are manifested in nearly every tissue and organ system. It is one of the first mediators to be synthesized in the course of an inflammatory event and is thought to initiate a cascade of effects. IL-1 is chemotactic for monocytes and lymphocytes (Hunninghake et al. 1986) and

mitogenic for fibroblasts (Bonin, Fici, and Singh 1989; Schmidt et al. 1982). In addition, IL-1 initiates collagen synthesis by increasing transcription of types I, III (Canalis 1986) and IV collagen (Matsushima et al. 1985). Therefore, fibrosis and deposition of abnormal proteins in tissues appears to be mediated, in part, by IL-1.

Tumor Necrosis Factor-α

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine that plays a pivotal role in immune and inflammatory reactions. It was first described as an endotoxin-induced serum protein that caused tumor-specific hemhorragic necrosis of tumor cells (Carswell et al. 1975). Although macrophages and monocytes are the major source of TNF-α (Mannel, Moore, and Mergenhagen 1980; Matthews 1981), other cell types can also synthesize this cytokine. TNF-α is identical to cachectin, the serum factor responsible for the wasting syndrome associated with chronic infections and cancer (Beutler et al. 1985). TNF-α is important in the initiation and subsequent remodeling phases of the inflammatory response. Studies have suggested that TNF-α is chemotactic for both PMNs and monocytes (Ming, Bersani, and Mantovani 1987), although this function may be indirectly mediated by TNF-α. TNF-α has also been reported to have growth stimulatory effects on fibroblasts (Thornton et al. 1990) and can induce angiogenesis(Leibovich et al. 1987). Overproduction of TNF-α is linked to chronic inflammatory states and fibrosis.

JE/MCP-1

The JE gene, first identified in mouse 3T3 cells, is a PDGF-inducible early response gene (Cochran, Reffel, and Stiles 1983). Unlike other early response

genes, JE encodes a secreted glycoprotein with cytokine-like properties. The human homologue of JE has been cloned and identified as monocyte chemotactic protein-1 (MCP-1) (Rollins et al. 1990; Yoshimura et al. 1989). JE/MCP-1 belong to a recently identified family of low molecular weight secreted proteins involved in the recruitment of cells during the inflammatory response that include IL-8 and monocyte inflammatory protein (MIP) (for a review, see Oppenheim et al. 1991). In vitro, JE is a potent monocyte chemoattractant and activating factor (Rollins, Walz, and Baggiolini 1991; Yoshimura and Leonard 1990; Yoshizuka et al. 1989). In vivo, JE causes monocyte accumulation (Zachariae et al. 1990) and induces monocyte-mediated tumoricidal activity (Rollins and Sunday 1991).

Because recruitment of cells, especially those of the monocyte and macrophage lineage, is critical to the outcome of a healing tissue, regulation of JE could ultimately affect the outcome of a wound healing response. Investigators have shown that JE is present in several models of lung fibrosis (Brieland et al. 1993; Flory, Jones, and Warren 1993) and in atherosclerotic lesions (Koch et al. 1993; Nelken et al. 1991; Takeya et al. 1993; Yla-Herttuala et al. 1991; Yu et al. 1992). Not surprisingly, macrophages are found to be the predominant cell types producing JE in these instances of aberrant wound healing (Koch et al. 1993). These data suggest that, while JE production may be critical for the healing wound, sustained production of JE, especially by resident macrophages, results in overhealing.

Fibrogenic Cytokines

In contrast to the above-mentioned cytokines, PDGF and TGF-β are thought to be highly fibrogenic, as they are able to directly trigger the

proliferation of fibroblasts and/or stimulate the production of connective tissue (for a review, see (Kovacs 1991)). Aberrant production of these mediators has been demonstrated in animal models of lung fibrosis (Kovacs and Kelley 1985). Additional studies have shown that these cytokines are produced locally, at the site of tissue damage in the lung (Kovacs, Oppenheim, and Young 1986) as well as in the peritoneal cavity of patients undergoing peritoneal dialysis (Shimakado et al. 1985) or intraabdominal cancer therapy (Kovacs and Urba, unpublished observations).

Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is a cationic, heat stabile protein of approximately 30 kD. Although PDGF was first discovered in the alpha granules of platelets (Shimakado et al. 1985), it has since been found in a wide variety of cell types, of both normal and transformed phenotype. These cell types include monocytes (Martinet et al. 1986), macrophages (Shimakado et al. 1985), endothelium (Ross, Raines, and Bowen-Pope 1986; Starksen et al. 1987), smooth muscle cells (Sjolund et al. 1988) and fibroblasts (Paulsson et al. 1987). Interestingly, macrophage-derived PDGF was first isolated from cells obtained from peritoneal dialysis patients (Shimakado et al. 1985). PDGF is the primary source of mitogenic activity in plasma for mesenchymal connective tissue-forming cells (Kohler and Lipton 1974; Ross et al. 1974; Rutherford and Ross 1976). In addition, it is highly chemotactic for mesenchymal cells (Grotendorst et al. 1981; Grotendorst et al. 1982; Seppa et al. 1982) and to a lesser degree PMNs and mononuclear cells (Deuel et al. 1982; Williams, Antoniades, and Goetzl 1983). PDGF not only stimulates collagen production (Canalis 1981), but

it also increases collagenase activity (Bauer et al. 1985). Thus, PDGF is thought to play a central role in tissue remodeling and wound healing.

Transforming Growth Factor-β

Transforming growth factor-β (TGF-β) is a homodimeric protein originally defined for its ability to reversibly induce a transformed phenotype and anchorage-independent growth of normal fibroblasts (Roberts et al. 1981). There five known isoforms of TGF-β. Of these, only types 1,2 and 3 have been demonstrated in mammalian tissue (type 4 is from chicken and type 5 is from frog). Although the biological activities of most of these isoforms are indistinguishable in vitro, their sites of synthesis and in vivo localization are often distinct. While platelets are the major storage site of TGF-β (Assoian et al. 1983), macrophages, lymphocytes and fibroblasts also produce TGF-β (Anzano, Roberts, and Sporn 1986; Assoian and Sporn 1986; Kehrl et al. 1986). TGF-β has been shown to stimulate fibroblast proliferation (Hill et al. 1986) and synthesis of extracellular matrix proteins, including collagen molecules I, II, V, fibronectin, and other various proteoglycans (Ignotz and Massague 1986; Madri, Pratt, and Tucker 1988; Varga, Rosenbloom, and Jimenez 1987). In addition, TGF-β is a chemoattractant for both fibroblasts and inflammatory cells both in vitro and in vivo (Mustoe et al. 1987; Postlethwaite et al. 1987; Roberts et al. 1986; Wahl et al. 1987). TGF-β-like activity has been found in several ovarian cancer cell lines (Berchuck et al. 1990) and in the peritoneal lavage fluid of ovarian cancer patients. Our laboratory has shown that the peritoneal lavage fluid from patients undergoing IL-2/LAK cell therapy contains both TGF-β and PDGF (Kovacs and Urba, unpublished observation) and that human peripheral blood lymphocytes and rat resident peritoneal macrophages stimulated with IL-

2 produce both cytokines (Kovacs et al. 1989; Kovacs and Neuman 1991; Kovacs, Stedum, and Neuman 1994). Thus, growth factors such as TGF-β and PDGF are present in the peritoneal cavity at sites adjacent to mesenchymal cells and may play a critical role in the development of peritoneal fibrosis.

The Immune-Endocrine Axis

It has become widely accepted that the endocrine and immune systems interact extensively to regulate each other (Grossman 1985). In response to stress, there is an activation of the hypothalamic-pituitary-adrenal axis leading to the output of glucocorticoids. Besides having dramatic effects on metabolism, these glucocorticoids have strong immunosuppressive and antiinflammatory properties. A less-studied set of steroid hormones with potentially significant effects on the immune system are the gonadal steroids. This lack of research is surprising, considering that it has long been known that a sexual dimorphism exists as far as the immune response is concerned (Ansar-Ahmed, Penhale, and Talal 1985). Furthermore, pregnancy causes a wide variety of immune function-altered events to occur (Carr 1990).

Steroid hormones are theorized to work by binding to nuclear receptors located within the cell (Figure 4) (Murdoch and Gorski 1991). In this model, free steroid dissociates across the plasma membrane and is transported to the nucleus where it binds to the unoccupied steroid receptor. Once bound to its ligand, the steroid receptor is now able to bind to specific regions of DNA, known as steroid response elements, and thereby affect transcription of target genes.

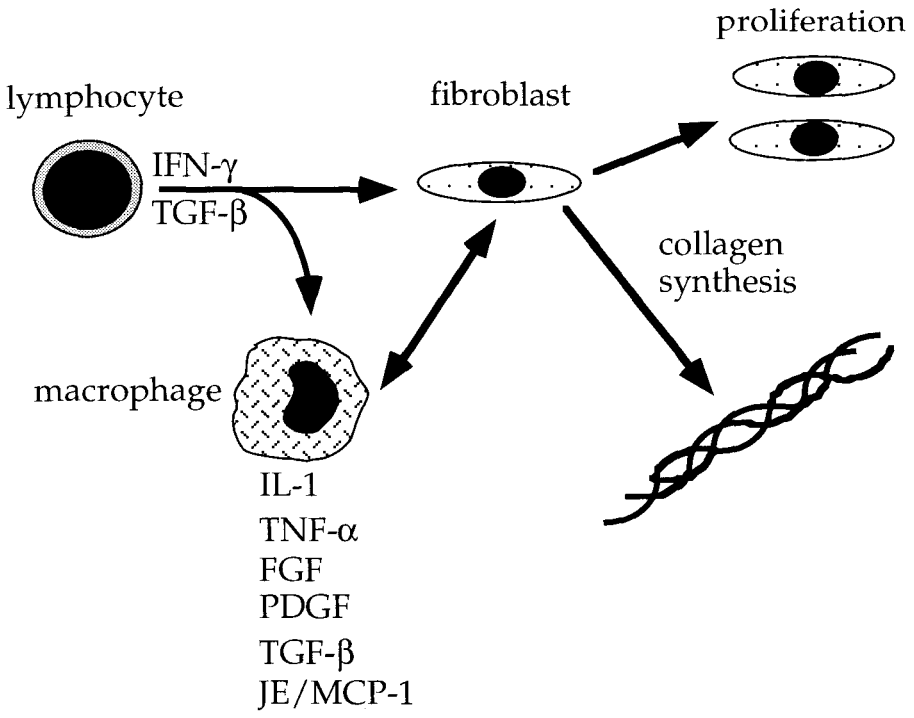

Figure 3. Immune Cell Populations Involved in the Wound Healing Response.

Glucocorticoids

Glucocorticoids have long been known to suppress immune functions and are thus thought to help regulate both the cellular and humoral immune responses under normal and stressing conditions. Physicians have used glucocorticoids in the clinical management of inflammatory and immunologic diseases for many years. One of the caveats of using glucocorticoids is their far-reaching and potentially harmful immuno-suppressive actions. Conversely, pathologic alterations in glucocorticoid secretion can seriously affect the immune response.

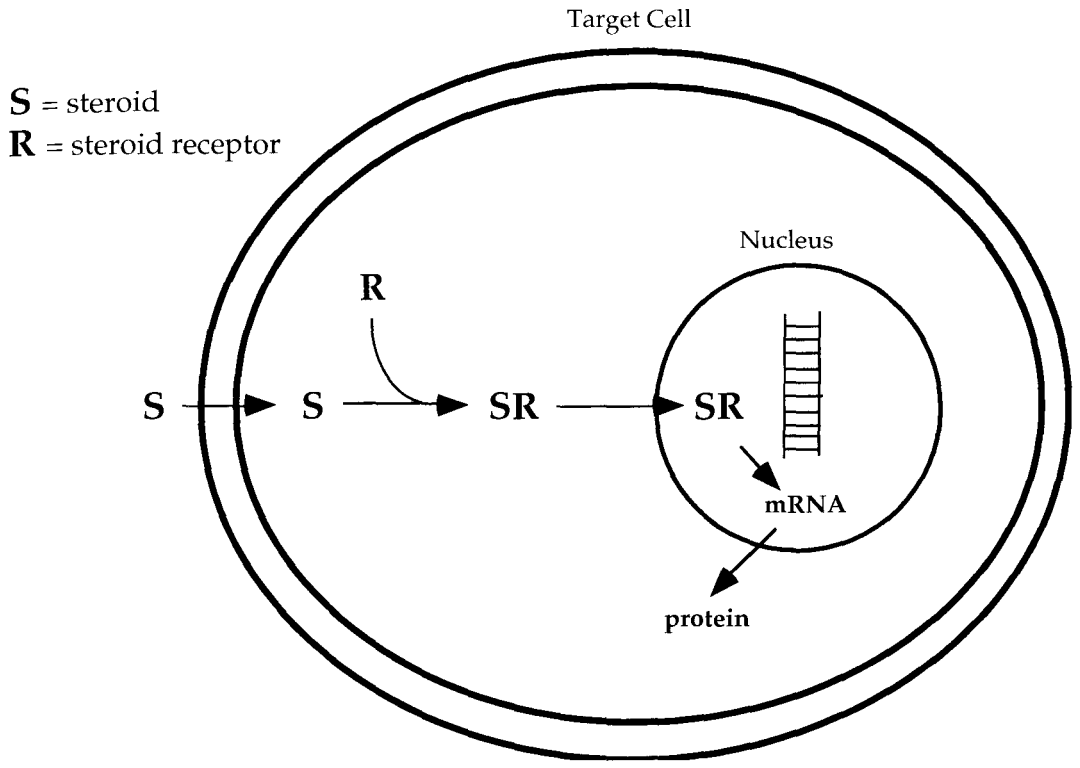

Figure 4. Proposed Mechanism of Action of Steroids.

Effects on Fibroblasts

As previously discussed, fibroblasts are responsible for the repair and maintenance of the extracellular matrix. Collagen is the major connective tissue protein synthesized by fibroblasts. Studies have shown that glucocorticoids inhibit collagen synthesis in normal and inflamed tissues (Cockayne et al. 1986). In addition, migration and proliferation of fibroblasts into wound sites are inhibited by glucocorticoids (Durant, Duval, and Homo-Delarche 1986). Because collagen is the major extracellular matrix protein in vertebrates, it is important that if glucocorticoids are administered for anti-inflammatory

purposes, that they are given at the lowest possible dose, so that inflammation is inhibited while minimizing the inhibitory effects of fibroblast collagen synthesis of normal connective tissues.

Effects on Macrophages

Glucocorticoids have profound effects on both macrophage function and activation (Guyre and Munck 1989). In vivo administration of glucocorticoids leads to a dramatic depletion and subsequent redistribution of monocytes from the periphery to the bone marrow (Hahn et al. 1980). Furthermore, cell-mediated immune reactions are generally inhibited (Weston, Mandel, and Yeckley 1973). Both inflammatory response and wound healing capabilities of macrophages are inhibited in vivo (Guyre and Munck 1989). These in vivo observations correlate with in vitro findings showing diminished functional response in macrophage antigen processing and presentation (Hirschberg et al. 1982), inhibition of monocyte chemotaxis (Rinehart et al. 1975), inhibition of phagocytosis (Shepard, Konish, and Stahl 1985), and suppression of lymphokine-induced giant cell formation (Galindo 1984). Furthermore, glucocorticoids also inhibit prostaglandin release (Wahl and Winter 1984). All of the above-mentioned phenomena are critical in the development and resolution of the inflammatory response.

Estrogen

Estrogen is essential for the development and maintenance of the female reproductive tract (Carr 1990). Estrogen in produced in the ovary during the reproductive years, in larger quantities at the onset of puberty and throughout menarche until menopause. Estrogens are synthesized by the aromatization of

androgens (dihydroepiandrostenedione and testosterone). Three estrogens are produced by the ovaries, two of which are produced in relative abundance. Of these, the most potent estrogen is estradiol-17β (E_2). Estrogen levels vary throughout the reproductive cycle and are under the control of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Estrogen secretion is low during the early follicular phase and increases prior to the midcycle gonadotrophin surge, peaking and then dropping off at the surge.

Estrogens stimulate DNA synthesis and cell proliferation in the female reproductive organs and other target tissue sites. In the reproductive tissues, estrogens are involved in the inflammatory and tissue remodeling events that occur throughout the menstrual cycle. For example, estradiol decreases the amount of collagenase produced by rat uterine cervical fibroblasts in culture (Sato et al. 1991). In addition, estrogen-progestogen therapy in postmenopausal women has been shown to decrease type III collagen production (Kashnikova, Grozdova, and Panasiuk 1991). Estradiol replacement for postmenopausal women is very effective at treating bone loss (Ralston, Russell, and Gowen 1990) probably due to its ability to inhibit the release of TNF-α, a potent stimulator of bone resorption. Furthermore, one of the factors associated with an increased risk in the development of cardiovascular disease in women is the onset of menopause. Epidemiological studies have shown that estrogen replacement reduces the risk of development of cardiovascular disease (Eaker and W.P.Castelli 1987; Knopp 1988; Paganini-Hill, Ross, and Henderson 1988; Stampfer et al. 1991; Sullivan et al. 1990) by poorly understood mechanisms.

Estrogen receptors have been found in monocytes/macrophages (Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986), lymphocytes (Cohen et al. 1983), mast cells (Cocchiara et al. 1990), and resident mesenchymal

cells (Malet et al. 1991), suggesting that estrogens can directly alter the immune response by modulating of both proliferation of these cell types and their cytokine production profile. Thus, there is a link between estrogen and connective tissue production and estrogen and the immune response.

Further evidence for the role of sex steroids in the fibrotic process is that connective tissue diseases, such as scleroderma and systemic lupus erythematosus, are significantly more prevalent in women of childbearing age than in postmenopausal women and men (Czirjak et al. 1989; Steen 1990; Steen and Medsger 1990). These disease states are often altered during pregnancy, when steroid hormone levels are markedly elevated. In addition, certain types of arthritis are modulated by female sex hormones (Holmdahl and Jansson 1988).

Only a handful of studies have reported on the effects of gonadal steroids on the expression of cytokine genes in purified immune cell populations. Hu and coworkers (Hu, Mitcho, and Rath 1988) showed that adherent peritoneal cells obtained from intact female rats secreted greater amounts of IL-1 than cells from male or ovariectomized (OVX) female rats. The increased production of IL-1 is restored following E_2 replacement in OVX animals. IL-1β mRNA expression in LPS-stimulated human monocytes (Polan et al. 1989) was elevated marginally in response to low physiological levels of E_2 and inhibited markedly after treatment with high doses of E_2. The doses required to diminish LPS-induced expression of IL-1β mRNA were 10^{-7} to 10^{-5}, well above the physiological range. The requirement for such high doses suggests that E_2 may not be utilizing specific high affinity receptors, but rather low affinity estrogen binding sites reported to be present on peripheral blood mononuclear cells (Ranelletti et al. 1988; Wada et al. 1992). At physiological levels, E_2 had marked

inhibitory affects on the production of TNF-α by human peripheral blood monocytes from postmenopausal women (Ralston, Russell, and Gowen 1990). Similar affects were not observed in monocytes obtained from men or premenopausal women. This difference may be due to the relative levels of estrogen receptors in men, premenopausal and postmenopausal women. Hence, a majority of the studies revealed that normal physiological levels of E_2 enhanced cytokine mRNA expression and high physiological (and pharmacological) doses turned off expression. Because of the design of these experiments, it is not clear whether the observed effects of E_2 on cytokine gene expression were direct or indirect.

A recent report identified the locations of four estrogen response elements (ERE), which serve to modulate gene expression, in the 5' flanking region of the murine interferon-γ (IFN-γ) gene (Fox, Bond, and Parslow 1991), showing for the first time that estrogen could have a direct effect on the control of expression of a cytokine gene. They also reported that estrogen acted synergistically with concavalin A to trigger expression of IFN-γ mRNA in murine splenocytes. In view of the pleiotropic effects of IFN-γ on the function of immune cells, including the activation of macrophages to produced a variety of cytokines, stimulation of IFN-γ production by E_2 could have marked stimulatory effects on the immune system.

Modulation of the production of these cytokines and connective tissue proteins by E_2 in vivo could have profound effects on the deposition of peritoneal adhesions (Figure 5). Estrogen may act at one or more levels to trigger connective tissue deposition. We and others have shown that estrogen triggers lymphocytes to produce IFN-γ ((Fox, Bond, and Parslow 1991); Kovacs and Mott, unpublished observation). Furthermore, elevated expression of IFN-γ

can trigger resident macrophages to produce proinflammatory and fibrogenic cytokines. These mediators, in turn, can enhance the proliferation of fibroblasts and the synthesis of collagen. In addition, since ER are present in circulating mononuclear cells (Wada et al. 1992; Weusten et al. 1986) and macrophages (Gulshan, McCruden, and Stimson 1990), estrogen may act directly on macrophage activation. Fibroblasts can be triggered by estrogen to produce both fibrogenic cytokines and connective tissue proteins. Thus, systemic elevation of estrogen could have both direct and indirect stimulatory effects on connective tissue production.

Investigation of the control of expression of cytokine genes and the role these mediators play in the development of peritoneal fibrosis is of immediate clinical relevance. In addition, furthering our understanding of the role of female sex steroids in this process will help to explain the mechanisms by which females are more susceptible to autoimmune diseases and connective tissue disorders. The results of these studies are also directly relevant to the control of aberrant wound healing, which is the hallmark of a variety of diseases including atherosclerosis and pulmonary fibrosis.

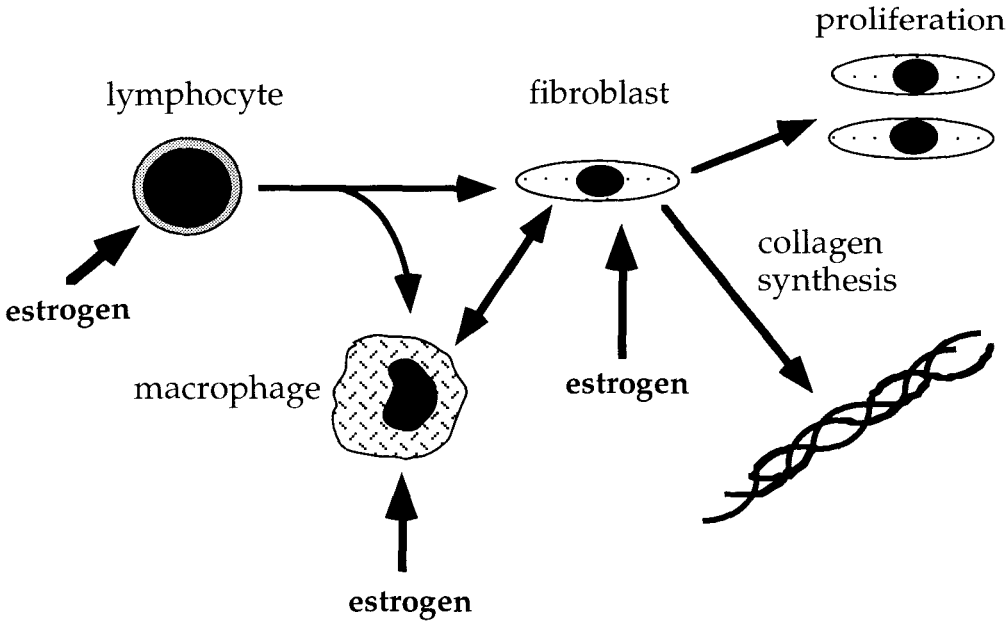

Figure 5. Potential Effects of Estrogen on Cells Involved in the Wound Healing Response.

CHAPTER 3

ABDOMINAL WALL THICKNESS AS A MEANS OF ASSESSING PERITONEAL FIBROSIS IN MICE

Abstract

Herein we describe a method for the quantitative assessment of connective tissue deposition within the peritoneal cavity. Female C57BL/6 mice (8-10 weeks) were given a single intraperitoneal injection of varying concentrations of talc (100 mg, 50 mg, 30 mg, 20 mg) in 1 ml of PBS or PBS alone. After 14 days, animals were killed. Adhesion formation was measured by the standard method of Myllarniemi et al. (Myllarniemi et al. 1966), namely a gross visual inspection of the peritoneal cavity. This analysis gave a crude assessment of connective tissue deposition in the abdominal cavity but did not allow one to distinguish more subtle differences between intermediate dosage groups. In addition, a histological evaluation was performed. For the latter method, portions of the abdominal wall of mice were fixed and processed for histological analysis using Masson's Trichrome stain which allows for differentiation of connective tissue components. The thickness of connective tissue between the parietal peritoneum and the underlying abdominal wall muscle was measured. A dose-dependent increase in connective tissue deposition was observed in talc-treated animals compared to saline control animals. A differential cell count of the peritoneal exudate cells (PEC) showed that there was no change in cell populations in talc treated animals (compared

to control animals). Given the above results, the measurement of connective tissue thickness was found to give the most accurate assessment of peritoneal fibrosis than other previously used methods.

Introduction

The peritoneum is an extensive serous membrane covering the entire abdominal wall of the body and the free surfaces of internal organs. It consists of a single layer of mesothelial cells that normally permit the viscera to glide easily against the abdominal wall and each other. Damage to these mesothelial cells results in the exposure of the underlying basement membrane. Leukocytes attracted to and stimulated by extracellular matrix components release immune mediators, or cytokines, that can either lead to repair of the mesothelium or trigger the overproduction of fibrous connective tissue (fibrosis). In extreme cases, thick sheets of scar tissue can constrict, resulting in irreversible alteration of organ structure and function. The factor(s) responsible for determining whether re-establishment of an intact mesothelium can take place prior to the production of excessive scar tissue is (are) unclear at present. Injury to the mesothelium can be triggered by a variety of conditions, including intraperitoneal immunotherapy (Steis et al. 1990; Urba et al. 1989), surgery (Fabri and Rosemurgy 1991), peritoneal dialysis (Daugirdas et al. 1986; Gandhi et al. 1980), or tumor cell growth (Frieman, Muller, and Pott 1990; Vlasveld et al. 1992).

These effects are likely due to the elaboration of mediators secreted by both resident peritoneal cells (primarily macrophages) and infiltrating inflammatory leukocytes in response to local activation. These mediators, including platelet-derived growth factor (PDGF) and transforming growth

factor-β (TGF-β), stimulate the proliferation of fibroblasts and the deposition of collagen (for a review, see Kovacs 1991), resulting in the permanent alteration of the peritoneal architecture. We have previously shown that supernatants from interleukin-2 (IL-2) stimulated leukocytes induce the proliferation of fibroblasts (Kovacs and Neuman 1991).

There is no established method for the assessment of peritoneal connective tissue deposition. Current methods for quantification of a fibrotic response are crude, at best. Thus, our aim was to develop a method by which peritoneal response to inflammatory stimuli could be critically evaluated. Talc (hydrous magnesium silicate) was used to initiate an inflammatory event, since this agent has long been known to cause adhesion formation (Eismann, Seelig, and Womack 1947; Lichtman et al. 1946). We examined several parameters as potential indicators of fibrosis. These included: (1) a gross visual inspection and scoring of the peritoneal cavity for adhesion formation; (2) a measurement of the connective tissue thickness between the abdominal wall muscle and mesothelium; and (3) a determination of the cell populations present in the abdominal cavity during this process. The resulting data show that measurement of connective tissue thickness gave the most accurate assessment of connective tissue deposition in comparison to the other methods.

Materials and Methods

Reagents

Talc (hydrous magnesium silicate, $H_2O_3Si_3/_4Mg_1$) was obtained from Sigma Chemical Co. (St. Louis, MO). Phosphate-buffered saline (PBS) was obtained from Gibco Laboratories (Grand Island, NY).

Animals

Female C57BL/6 mice, 8-10 weeks of age, were obtained from Harlan Laboratories (Indianapolis, IN). Animals were maintained on a 12 hour light/dark cycle and provided with food and water ad libidum. All experimental procedures were performed in accordance with Loyola University Animal Research Facility guidelines.

Induction of Peritoneal Adhesions

Suspensions of talc in PBS (20, 30, 50, or 100 mg of talc per 1 ml of PBS) or PBS alone were injected intraperitoneally into mice. After 14 days, animals were sacrificed for analysis of peritoneal abnormalities.

Collection and Analysis of Peritoneal Cells

To assess the abdominal cell population, peritoneal lavage was performed by instilling 5 ml of chilled calcium/magnesium-free PBS into the peritoneal cavity. The peritoneal cavity was carefully opened and the fluid withdrawn. The lavage fluid was centrifuged at 1000 X g for 10 minutes and the resulting cell pellet resuspended in RPMI 1640 with 10% fetal bovine serum at a concentration of 500,000 cells/ml. 200 ml of this suspension was cytocentrifuged for 10 minutes at 550 rpm. Slides were removed, allowed to air dry and then fixed in methanol for 10 minutes. The cells were stained with Modified Wright Giemsa stain (Sigma) for differential analysis. A total of 300 cells were counted per animal.

Analysis of Peritoneal Cavity and Adhesion Scoring

The peritoneal cavity was opened and the number of adhesions assessed according to a scale developed by Myllarniemi et al. (Myllarniemi et al. 1966) A score of 1 indicated no adhesions, 2 indicated one to three separate adhesions, 3 indicated three or more distinctly separate adhesions and 4 indicated diffuse, sheet-like adhesions.

Histological Analysis of Peritoneal Wall Connective Tissue Deposition

The right upper quadrant of the abdominal wall was removed and placed in 10% buffered formalin. Formalin-fixed tissue was dehydrated in varying grades of ethanol and xylene and embedded in paraffin. Tissue sections were cut at 4 mm and stained for connective tissue components using Masson's Trichrome stain (Luna 1968). This procedure differentially stains connective tissue elements, such as collagen and muscle tissue, blue and red, respectively. Sections were deparaffinized and hydrated to distilled after, after which they were mordanted in Bouin's solution for one hour at 56^{o}C, cooled and rinsed in distilled water. This was followed by staining with Biebrich scarlet-acid fuchsin solution for 2 minutes and a rinse in distilled water. Sections were next placed in a phosphomolybdic-phosphotungstic acid solution for 10 minutes and rinsed in distilled water. After staining in aniline blue solution for one minute and a subsequent rinse in distilled water, slides were placed in glacial acetic acid for one minute. Finally, slides were rinsed, dehydrated through a series of alcohol gradations, cleared in xylene and mounted with Accu-Mount (Baxter Healthcare Products; McGaw Park, IL).

Sections were examined by light microscopy and the thickness of the connective tissue between the fragmented mesothelium and the abdominal muscle measured in mm. Each tissue sections was measured at ten random locations; five tissue sections were examined per animal. Each treatment group consisted of 5-7 animals. Measurements of trichrome stained connective tissue excluded spaces which were occasionally present and are presumably a result of sectioning artifacts.

Statistical Analysis

Data are expressed as mean + SEM where appropriate. Data on peritoneal membrane thickness were compared using a one-way ANOVA followed by Tukey's honestly significant difference.

Results

Adhesion Scores of Mice Treated with Various Concentrations of Talc

All of the animals receiving an intraperitoneal injection of talc developed some form of adhesions. Table I shows the adhesion score rating for the different treatment groups. Control mice did not develop adhesions, whereas animals receiving the highest dose (100 mg of talc) had diffuse continuous adhesions. Mid-range doses yielded intermediate levels of adhesions. While this scoring method gave a quick analysis of the fibrotic response, it did not permit the assessment of subtle differences, especially between the midrange treatment groups.

TABLE I: Peritoneal Adhesion Scores in Talc-Treated Mice

TREATMENT[a]	SCORE[b]
Saline	1
20 mg Talc	2
30 mg Talc	3
50 mg Talc	3
100 mg Talc	4

[a]Mice received a single intraperitoneal injection of varying doses of talc (n = 7).

[b]Adhesions were scored by gross visual inspection fo the peritoneal cavity. A score of 1 = no adhesions; 2 = 1 to 3 separate adhesions; 3 = some (3 or more separate) adhesions; 4 = abundant, diffuse, or sheet-like adhesions.

Quantitative Assessment of Connective Tissue Deposition on the Abdominal Wall

Figure 6 shows photomicrographs of representative sections taken from animals 14 days after receiving a single intraperitoneal injection of talc. Upon histologic examination, an increase in connective tissue was noted in animals receiving the upper talc dosages. This increase appeared as a thickening between the abdominal wall surface (striated muscle) and the fragmented mesothelial layer of the peritoneum. Connective tissue deposition was not observed in animals treated with saline alone.

Measurement of trichrome stained sections revealed that there was a dose-dependent increase in abdominal wall connective tissue thickness. Figure 7 shows a comparison of the treatment groups and their respective thicknesses. The 50 and 100 mg talc treatment groups differed significantly from the saline control animals ($p<0.0001$). Although the 30 mg talc treatment group did not

significantly differ from the saline control animals in the statistical analysis there was a definite trend noted between this group and the saline controls. Thus, these measurements revealed quantitative differences between groups that were not apparent using crude adhesion scores alone.

Peritoneal Cell Populations

While connective tissue thickness was markedly altered in talc-treated animals, resident peritoneal cell populations did not change in comparison to control animals. Figure 8 shows the percentage of macrophages, lymphocytes, and polymorphonuclear cells (PMNs) present in the lavage fluid of each treatment group with the macrophage population accounting for the majority of

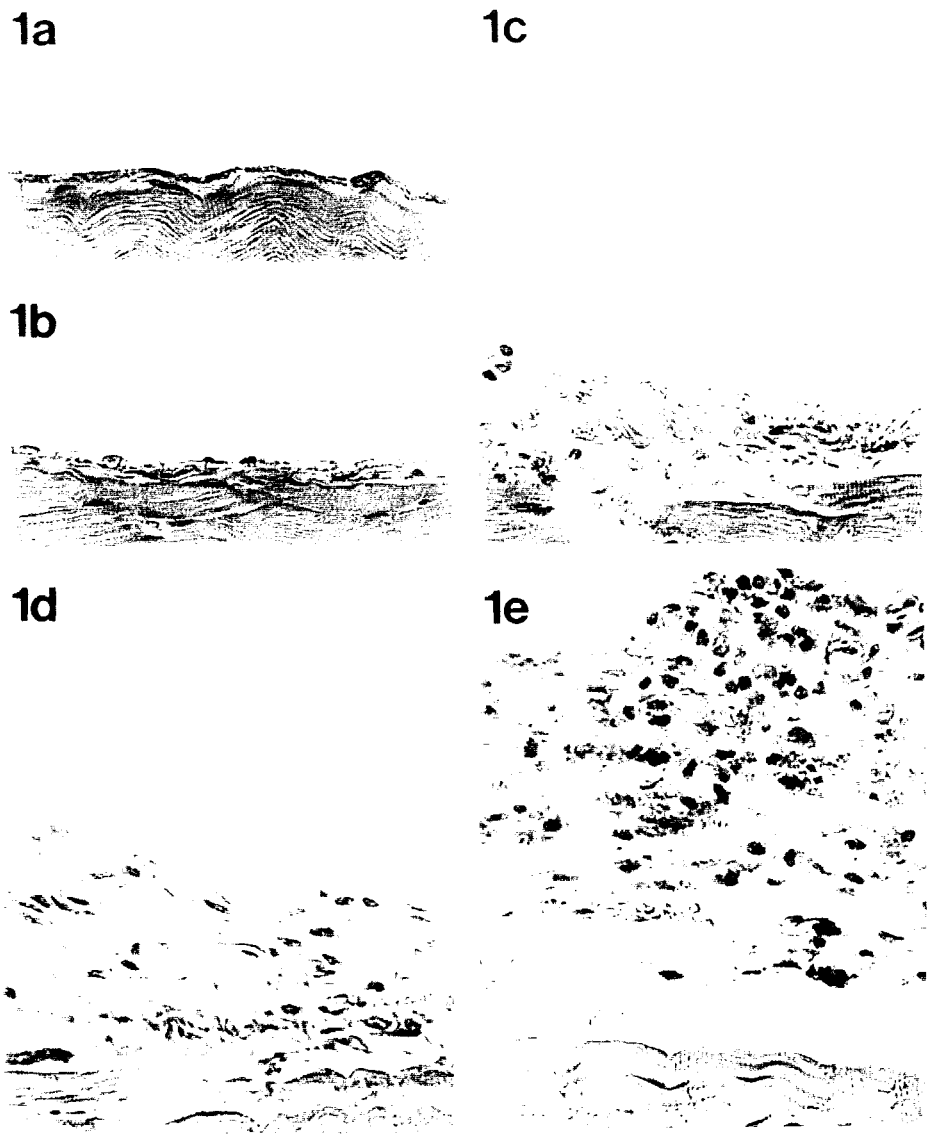

Figure 6. Thickness of Connective Tissue on Peritoneal Surface of Abdominal Muscle 14 Days After Treatment with Talc. Shown are representative sections of trichrome-stained abdominal wall with increasing amounts of connective tissue between the musculature and the fragmented peritoneal mesothelium. a is from an animal treated with saline alone; b is from an animal that received 20 mg of talc; c: 30 mg of talc; d: 50 mg of talc; and e: 100 mg of talc (magnification 500X).

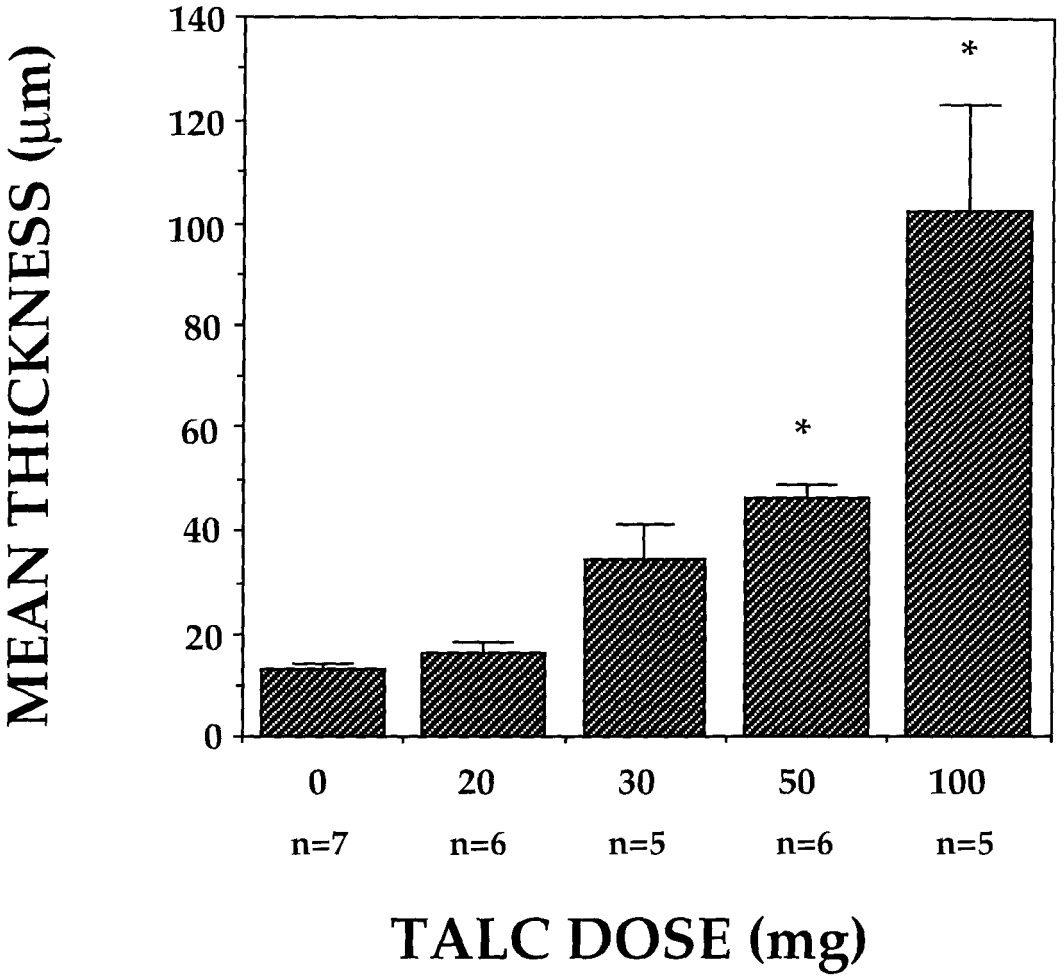

Figure 7. Peritoneal Wall Connective Tissue Thickness 14 Days After Treatment. Thickness, in mm, was assessed using Masson's Trichrome stain (see Materials and Methods section). Doses of 50 and 100 mg talc were significantly different ($p<0.001$) from saline treated animals.

cells in the peritoneal cavity. Initial attempts were made to analyze the total number of PEC in all treatment groups. Because the adhesions literally walled off areas in the peritoneal cavity, collection of PEC was difficult. This rendered quantitative assessment of the total number of cells impossible.

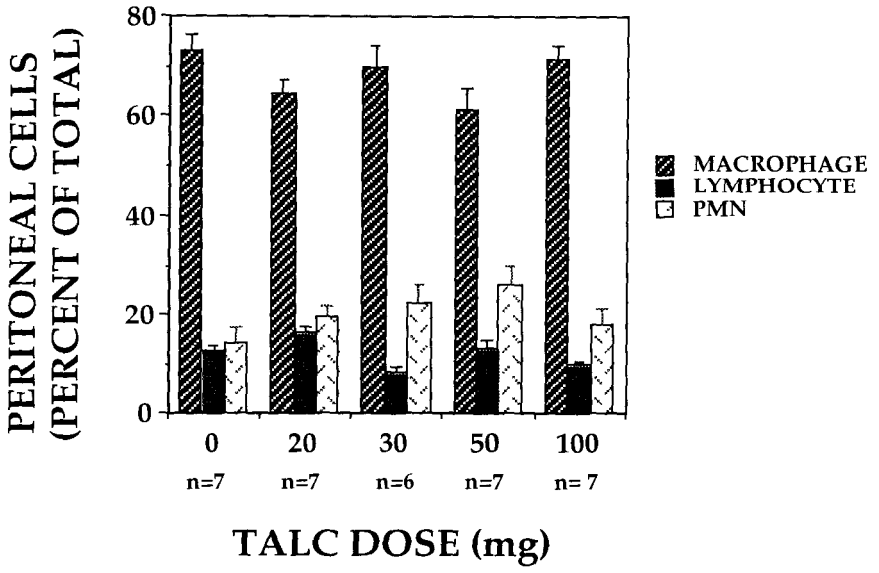

Figure 8. Differential Cell Count of Peritoneal Cells 14 Days After Treatment. No significant difference was observed between treatment groups.

Discussion

The intraperitoneal injection of talc resulted in a fibrotic response, causing both the development of peritoneal adhesions (Table I) and an increase in connective tissue deposition at the border between the peritoneal mesothelium and the intraabdominal wall (Figure 6). We observed an increase in the amount of connective tissue elements, including extracellular matrix and mesenchymal cells, within the submesothelial space, in accordance with previous studies (Renvall, Lehto, and Penttinen 1987). This reaction appeared to be dose-dependent, with the higher doses of talc (50 and 100 mg) causing a significantly greater thickness of connective tissue than saline alone ($p<0.001$). The use of connective tissue thickness measurements to assess the fibrotic

response gave a much more quantitative analysis of the degree of connective tissue deposition in the peritoneal cavity than the adhesion score method of Myllarniemi et al. (Myllarniemi et al. 1966) or than analysis of PEC composition (Figure 8).

In animals, the cells in the peritoneal fluid normally consist of 25-70% macrophages (Casciato, Goldberg, and Bluestone 1976). We found this to be true for our saline control animals. However, at 14 days after talc treatment, animals did not show any changes in peritoneal cell composition. This suggests that, while the differential population of resident cells remained the same, the production of fibrogenic cytokines by those cells may be altered. Since the macrophage population constitutes the majority of cells within the peritoneal cavity and macrophages are intimately involved in wound healing/tissue remodeling (Leibovich and Ross 1975), it is likely that resident peritoneal macrophages are responsible for the elaboration of fibrogenic cytokines in the study described herein.

It was our purpose to develop a more accurate method of assessing the fibrotic response within the peritoneal cavity. Peritoneal fibrosis is a frequent complication in both disease states and therapy regimens and yet there is no standard method of assessing the degree of adhesions. Until now, grading scales were the only means of rating connective tissue deposition. While these types of ratings are helpful, they do not delineate the more subtle differences. Our method provides for more distinction between treatment groups. In addition, this method has applications for in vivo assessment of topically applied therapeutic materials, such as Interceed (TC-7, oxidized regenerated cellulose; Diamond et al. 1991; Linsky et al. 1987)) a surgical adjuvant that was developed for prevention of adhesion formation. An animal could be treated

with Interceed (or other potential antifibrotic agents) on one side of the peritoneal cavity with the other side serving as an internal control, allowing for a quantitative in vivo evaluation of the tissue repair process.

CHAPTER 4

ESTROGEN REGULATION OF ABDOMINAL CONNECTIVE TISSUE DEPOSITION

Abstract

Estrogen's involvement in inflammation and wound healing is poorly understood. To examine the role of estrogen in peritoneal adhesion formation, we gave ovariectomized female C57BL/6 mice time-release pellets containing placebo , 0.05 mg 17β-estradiol (low E_2), or 5 mg 17β-estradiol (high E_2) before i.p. injection of talc in saline or saline alone. Analyses of abdominal wall connective tissue thickness and peritoneal cell populations were performed. Talc-treated mice receiving low and high E_2 replacement had a decreased amount of abdominal connective tissue deposition (29% and 65% decrease, respectively) as compared with talc-treated mice receiving placebo pellets. At high E_2 replacement, the difference in connective tissue deposition was significantly statistically ($p< 0.01$). Immunohistochemical analysis revealed that the number of macrophages in adhesion tissue was proportionate to the amount of connective tissue present, regardless of the circulating levels of E_2. Northern blot analysis of abdominal wall tissue showed that five of six talc-treated animals given placebo expressed mRNA for the murine monocyte chemoattractant protein-1 (MCP-1), JE. Conversely, only one of five talc-treated animals that received E_2 replacement expressed JE/MCP-1 mRNA, suggesting that the hormone may inhibit connective tissue deposition by altering the

production of chemotactic factors. Furthermore, E_2 suppressed talc-induced expression of JE/MCP-1 mRNA in murine macrophages. Since macrophages play a central role in the wound healing process, these studies suggest that E_2 inhibition of adhesion formation could be mediated by suppressing macrophage activation and/or recruitment to inflammatory sites.

Introduction

The sexual dimorphism of the immune response is well-documented (Ansar-Ahmed, Penhale, and Talal 1985; Grossman 1985; Schuurs and Verhuel 1990), yet the role the sex steroid hormone, estrogen, plays in the regulation of immune cell functions is only beginning to be understood. Connective tissue disorders and autoimmune diseases, such as scleroderma, lupus erythematosus, and rheumatoid arthritis are more prevalent in women of reproductive age than in women of all ages as well as in men and the severity of these disease states is often altered during pregnancy (Czirjak et al. 1989; Holmdahl and Jansson 1988; Steen 1990; Steen and Medsger 1990) . Elevated levels of circulating estrogen prevent certain pathologic conditions and can even trigger reversal of disorders. This is best demonstrated in post-menopausal women receiving estrogen replacement therapy, where hormonal replacement prevents bone loss (Christiansen 1993) and reduces the risk of coronary artery disease (Knopp 1988). These conditions are mediated by local activation of inflammatory and immune cells. Since these immune cell populations, including monocytes, macrophages, lymphocytes, and mast cells, express estrogen receptors (Cocchiara et al. 1990; Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986), it suggests that the hormone could directly affect functions of these cells, including cytokine production.

Fibrosis, or adhesion formation in the abdomen, is an end phase in the inflammatory response that can cause a multitude of disorders such as bowel obstruction (Krebs and Goplerud 1987) and infertility (Haney 1993). As in other systems, the deposition of abdominal connective tissue involves a cascade of events. Macrophages are recruited into the site of injury, where they secrete a host of mediators that in turn trigger the proliferation of fibroblasts and the synthesis and secretion of extracellular matrix proteins, such as collagen (for reviews see (Kovacs 1991; Kovacs and Pietro 1994)). It is well established that glucocorticoids have an inhibitory effect on the wound healing process by suppressing collagen synthesis (Cockayne et al. 1986; Cutroneo, K.M. Sterling, and Schull 1986; Walsh, LeLeiko, and K.M. Sterling 1987) and cytokine production (Beutler et al. 1986; Kawahara, Deng, and Deuel 1991; Lee et al. 1988; Mukaida et al. 1991; Snyder and Unanue 1982; Staruch and Wood 1985; Waage, Slupphaug, and Shalaby 1990). However, the effects of gonadal steroid hormones on connective tissue deposition and the wound healing process have yet to be examined.

Since estrogen modulates inflammatory responses in other systems, we hypothesized that it could alter the deposition of connective tissue in an in vivo model of peritoneal adhesion formation (Frazier-Jessen and Kovacs 1993), in which a suspension of talc was administered intraperitoneally. Talc from surgical gloves has been shown to cause adhesions following abdominal surgery (Henderson et al. 1978; Kus et al. 1979; Sheikh et al. 1984) and serves a reliable stimulus to trigger adhesion formation (Frazier-Jessen and Kovacs 1993). In this report, we show that estrogen suppresses connective tissue deposition in a dose-dependent manner and that this might be mediated

through inhibition of migration of resident peritoneal macrophages into the damaged tissue site by E_2.

Materials and Methods

Reagents

Talc (hydrous magnesium silicate, $H_2O_3Si_{3/4}Mg_1$), 17-β-estradiol (E_2), hydrogen peroxide, and diaminobenzidine tablets were obtained from Sigma Chemical Co. (St. Louis, MO). Brewer's thioglycolate and LPS were obtained from DIFCO (Detroit, MI). The F4/80 rat monoclonal antibody was a kind gift from Dr. Gary Wood (University of Kansas; Kansas City, KS). Normal donkey serum, biotinylated donkey anti-rat IgG, and purified rat IgG were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The Vectastain Elite ABC immuno-peroxidase kit was obtained from Vector Laboratories, Inc. (Burlingame, CA). 17β-estradiol (E_2) time-release pellets and placebo pellets were obtained from Innovative Research of America (Toledo, OH). The E_2 pellets were designed to release a total of 0.05 mg (low) or 5 mg (high) of E_2 over 21 days. All tissue culture reagents and random primer labeling kit were obtained from Gibco/BRL (Grand Island, NY).

Ovariectomy and E_2 Replacement

Female C57BL/6 mice, 8 to 10 weeks of age, were obtained from Jackson Laboratories (Bar Harbor, ME). All experimental procedures were performed in accordance with Loyola University Animal Research Facility guidelines. Ovariectomy (OVX) was performed to eliminate the major source of endogenous estrogens. Immediately following surgery, 21-day time-release

pellets containing either 0.05 or 5.0 mg of E_2 (the most biologically active form of estrogen), or vehicle alone, were implanted subcutaneously on the back according to manufacturer's directions. These concentrations of E_2 were chosen to deliver low physiologic levels (20 to 30 pg/ml; low E_2) and high pregnancy levels (2000 to 3000 pg/ml; high E_2), respectively, as described by the manufacturer (Bain et al. 1993; Langdon et al. 1993). In mice, the upper limit of normal for ciculating preovulatory E_2 levels is 200 to 300 pg/ml.

Animals were allowed to recover from surgery for 7 days before the induction of peritoneal adhesion formation. This time point allowed steroid hormone levels to equlibrate, which, according to the manufacturer, occurs within 48 hours of pellet implantation, and permitted complete healing of the peritoneal mesothelial lining, which takes less than 1 week (Ellis, Harrison, and Hugh 1965; Hubbard et al. 1967). The latter point is of importance to the study, because we wanted to insure that the surgical intervention into the abdominal cavity (required for OVX) did not interfere with pertioneal adhesion formation.

Induction of Adhesion Formation

Adhesion formation was induced by a single i.p. injection of a suspension of 30 mg of talc in saline (or saline alone as a control), as previously described (Frazier-Jessen and Kovacs 1993). Fourteen days after talc or saline injection, animals were killed for collection of resident peritoneal cells, tissue smaples of abdominal wall, and uteri. Uterine weight measured at the completion of the study was used as an indication of systemic estrogen delivery.

Collection and Analysis of Peritoneal Cells

To assess changes in peritoneal cell populations following E_2 and talc treatment, peritoneal lavage was performed as previously described (Frazier-Jessen and Kovacs 1993). Peritoneal cells were collected by cytocentrifugation, fixed in methanol, and stained with Modified Wright Giemsa stain for differential analysis. A minimum of 300 cells was counted per animal.

Histological Analysis of Peritoneal Wall Connective Tissue Deposition

The right upper quadrant of the abdominal wall was removed and placed in 10% buffered formalin, sectioned, and stained for connective tissue components using Masson's Trichrome stain as previously described (Frazier-Jessen and Kovacs 1993). Thickness of the connective tissue between the fragmented mesothelium and the abdominal muscle was measured in microns. Each 4-μm tissue section was measured at 10 random locations; five tissue serial sections were examined per animal. Measurements of trichrome-stained connective tissue excluded spaces that were occasionally present and are presumed to result from sectioning artifacts.

Immunohistochemical Analysis of Macrophages in Abdominal Wall Adhesion Tissue

The left upper quadrant of the abdominal wall was placed in O.C.T. compound (Miles Laboratories, Elkhart, IN), flash-frozen and stored at -70°C. Six micron sections were fixed in 4% paraformaldehyde and incubated with 50% normal donkey serum for 30 minutes to reduce nonspecific staining. The sections were then incubated sequentially with the monoclonal rat anti-mouse

macrophage antibody, F4/80, or with purified rat IgG (as a control antibody) for 1 hour and then with biotinylated donkey anti-rat IgG for 30 minutes. Endogenous peroxidase activity was blocked by exposure to 0.3% hydrogen peroxide before incubation with Vectastain Elite ABC immunoperoxidase kit. Horseradish peroxidase enzyme activity was detected by immersing the slides in 0.45 mg/ml diaminobenzidine (DAB) in buffer containing 0.15M NaCl, 0.05M Tris, and 0.07% H_2O_2. Sections were counterstained in methyl green and observed by bright field microscopy. The number of positive staining cells per given area was counted, using National Institutes of Health Image Analysis software, and expressed as number of cells/μm^2. Five areas per section were measured, with two sections for placebo/talc-treated animals, and three for high E_2/talc-treated animals. Because of the limited amount of connective tissue in saline-treated mice, assessment of immunoreactive cells was not performed on those animals.

Isolation and Culture of Thioglycolate-Elicited Murine Peritoneal Macrophages

Cycling female C57BL/6 mice were injected i.p. with 1.5 ml of 10% Brewer's thioglycolate (Difco), as previously described (Radzioch, Bottazzi, and Varesio 1987). Four days later, animals were killed and peritoneal lavage was performed, and cells were plated at 2 X 10^6 cells/ml of phenol red-free RPMI 1640 medium with 2% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2mM L-glutamine, as previously described (Frazier-Jessen and Kovacs 1995). Two hours after plating, medium containing nonadherent cells was removed and replaced with fresh medium. The remaining cells were >95% macrophages, as determined by Giemsa stain analysis. Macrophage

cultures were rested for 48 hours before stimulation to diminish expression of cytokine genes triggered by adherence to tissue culture plastic (Fuhlbrigge et al. 1987).

After resting, macrophages were cultured in the presence or absence of a talc suspension in PBS with or without E_2 for 8 hours, unlesss otherwise specified. The concentrations of E_2 (3-3000 pg/ml) were comparable with normal circulating levels in female mice and those attained in in vivo experiments using OVX animals given E_2 replacement. Stock solutions of E_2 were prepared in ethanol. Since ethanol is known to alter membrane permeability that may affect activation of second messenger pathways, care was taken to insure that effects of the steroid hormone were due to the hormone itself, rather than the solvent. In all experiments, final concentrations of ethanol did not exceed 0.0006%.

Parallel studies were performed using the ANA-1 murine macrophage cell line (obtained from Dr. Luigi Varesio, Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD). Forty eight hours before stimulation, ANA-1 macropahges were plated at 5 X 10^5 cells/ml in phenol red-free RPMI 1640 medium with 2% fetal bovine serum in the absence of exogenous stimulants, as previously described (Frazier-Jessen and Kovacs 1995). Cells were harvested for RNA isolation after 8 h of incubation, unless otherwise specified.

RNA Isolation and Northern Blot Analysis

Total RNA from homogenized abdominal wall tissue was isolated by a single-step guanidinium thiocyanate-phenol-chloroform method (RNAzol B; Cinna Biotex, Houston, TX) according to manufacturer's specifications.

Northern blots, prepared as previously described (Kovacs, Oppenheim, and Young 1986), were hybridized with ^{32}P-labeled cDNA probes for murine JE (obtained from the American Type Culture Collection, Rockville, MD) and glyceraldehyde-3-phospate dehydrogenase (G3PDH; from Dr. Hans-Martin Jack, Loyola University Chicago, Maywood, IL) using a random primer kit. Eithidium bromide staining of agarose gels was performed to demonstrate even loading of samples. Quantitation of RNA signals was performed by scanning densitometry using Ambis Quantprobe Software version 3.02 (Ambis Systems, Inc., San Diego, CA).

Statistical Analyses

Data are expressed as mean ± SEM. Data on uterine weight, peritoneal membrane thickness, and cell populations were compared using a two-way analysis of variance. A p value < 0.05 was considered to be significant statistcally. Studies were performed with a minimum of six animals per group, unless otherwise stated.

Results

Uterine Weight of Ovariectomized Animals With or Without E_2 Replacement

Uterine weight has previously been reported to serve as an accurate assessment of systemic estrogen exposure in OVX mice (Butterstein, Damassa, and Sawyer 1980; Gee et al. 1984; Mobbs et al. 1985). In the absence of circulating estrogen (OVX with placebo pellets), uterine weight is low, as shown in Table II. These values are comparable with the reduced uterine weight

observed in 6 week old C57BL/6 mice that averaged 19 ± 0.8 mg 2 weeks after OVX (Jesionowska, Karelus, and Nelson 1990). Physiologic replacement of E_2 in OVX animals results in a three- to fourfold increase in uterine weight over OVX animals that do not receive E_2 replacement ($p < 0.01$). These uterine weights in E_2-treated mice were higher than the levels reported for uterine weight in 6 week old C57BL/6 mice that ranged from a low of 52 ± 3.3 mg during midestrus to a high of 88 ± 8.5 during proestrus (Jesionowska, Karelus, and Nelson 1990). However, they are consistent with other studies performed in C57BL/6 (Gee et al. 1984; Mobbs et al. 1985) and other strains of mice (De and Wood 1990). In addition, high E_2 replacement stimulates a significant increase in uterine weight, but to a lesser extent than low physiologic E_2

TABLE II: Uterine Weight of Ovariectomized Mice Given E_2 Replacement With or Without i.p. Talc Administration

	Uterine Weight (mg)[b]	
E_2 Status[a]	Saline	Talc
placebo	35.75 ± 8.25 (n=4)	39.33 ± 10.28 (n=6)
low E_2	145.33 ± 5.81 (n=3)*	111.00 ± 7.77 (n=5)*
high E_2	113.6 ± 9.56 (n=3)*	89.86 ± 3.40 (n=7)*

Data are shown as mean + SEM.

[a]Mice were ovariectomized and implanted with pellets that released a total of 0.05 mg E_2 (low E_2), 5 mg E_2 (high E_2) or vehicle (placebo) over a 21-day period.

[b]Mice were injected i.p. with talc (30 mg in 1 ml saline) or saline 7 days post-ovariectomy/E_2 replacement. Fourteen days later, uteri were dissected out and weighed for verification that E_2 doses were within reported appropriate ranges. *$p < 0.01$ from placebo-treated animals.

replacement ($p < 0.01$), which has been reported previously by others (Bergman et al. 1992; Jesionowska, Karelus, and Nelson 1990). This difference is attributed to suppression of cycling frequency in mice given high E_2 levels relative to those treated with lower levels of the hormone, as previously reported (Bain et al. 1993; Langdon et al. 1993). Finally, we also observed that the uterine weights from talc-treated OVX animals given E_2 replacement were less than the weight of uteri in saline-treated mice, as there is no difference in the body weight of the animals in the treatment groups. It may be attributed to the presence of adhesions on the uteri of the talc-treated mice that restricted the aqueous weight gain of the organ; however, histologic analysis of the uteri would need to be performed to confirm this.

Assessment of Connective Tissue Deposition in the Abdominal Cavity

Data from representative trichrome-stained abdominal wall sections obtained from mice injected with either talc or saline are shown in Figure 9. Normally, the peritoneum consists of a single layer of mesothelial cells and their underlying basement membrane (Figure 9a). Animals injected with saline alone did not develop peritoneal membrane thickening, regardless of E_2 replacement. Injection of talc causes disruption of the peritoneal mesothelium and thickening of the underlying connective tissue (Figure 9b). This connective tissue consists primarily of inflammatory cells, fibroblasts, and collagen. Less connective was observed in talc-treated animals given low and high E_2 replacement (Figure 9, c and d, respectively) than talc-treated mice given placebo. Our quantitative assessment of peritoneal connective tissue thickness revealed that, in OVX/placebo mice, talc treatment triggered a fourfold increase in peritoneal

membrane thickening as compared with saline-treated controls ($p < 0.01$; Figure 10). Peritoneal connective tissue deposition in talc-treated animals receiving low E_2 replacement and high E_2 replacement was diminished markedly (29% and 65%, respectively) when compared with talc-treated mice given placebo pellets. At high physiologic E_2 replacement, this decrease in connective tissue accumulation was signficant statistically ($p < 0.01$).

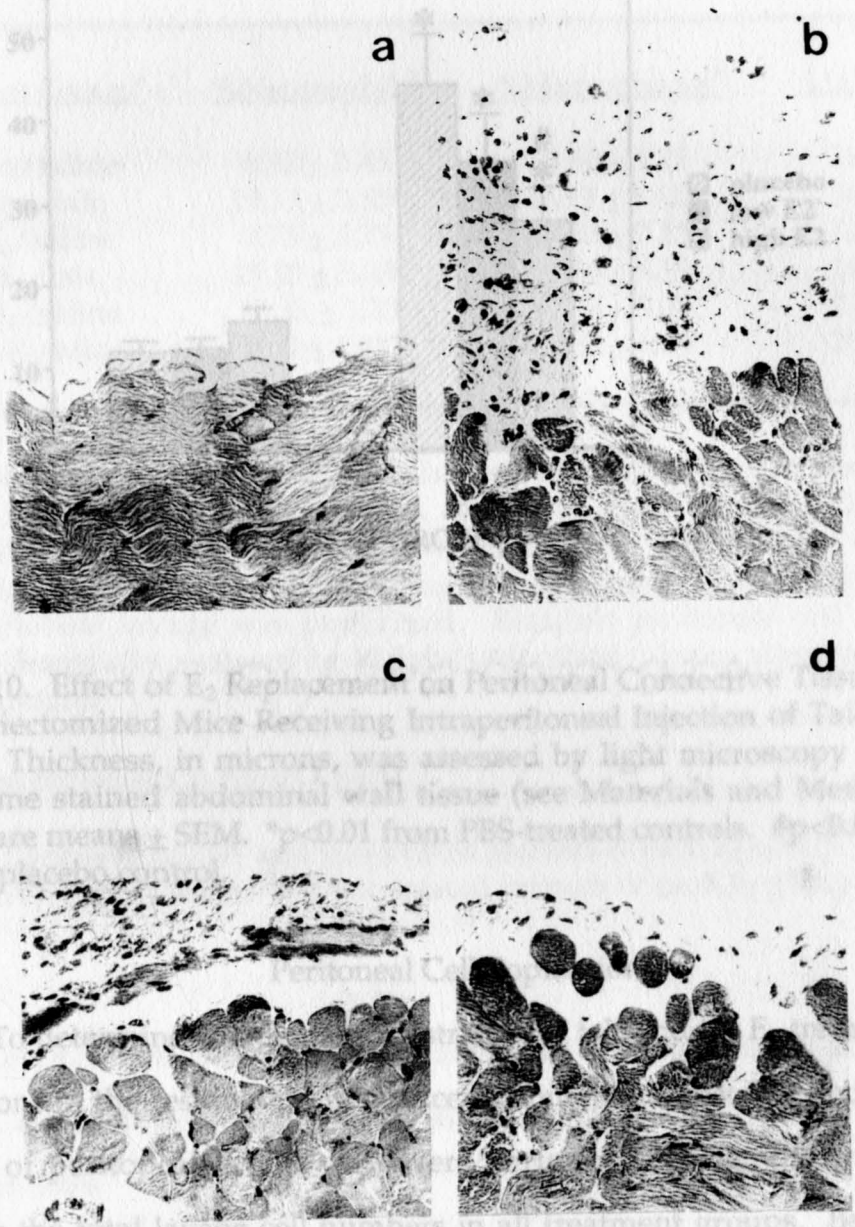

Figure 9. Histological Assessment of Peritoneal Connective Tissue Thickness in Talc-Treated, Ovariectomized Female C57BL/6 Mice Given E_2 Replacement. Representative trichrome-stained sections of abdominal wall: (a) no E_2 replacement (placebo), PBS; (b) placebo with talc; (c) low E_2 replacement with 30 mg talc; (d) high E_2 replacement with talc (magnification 600X).

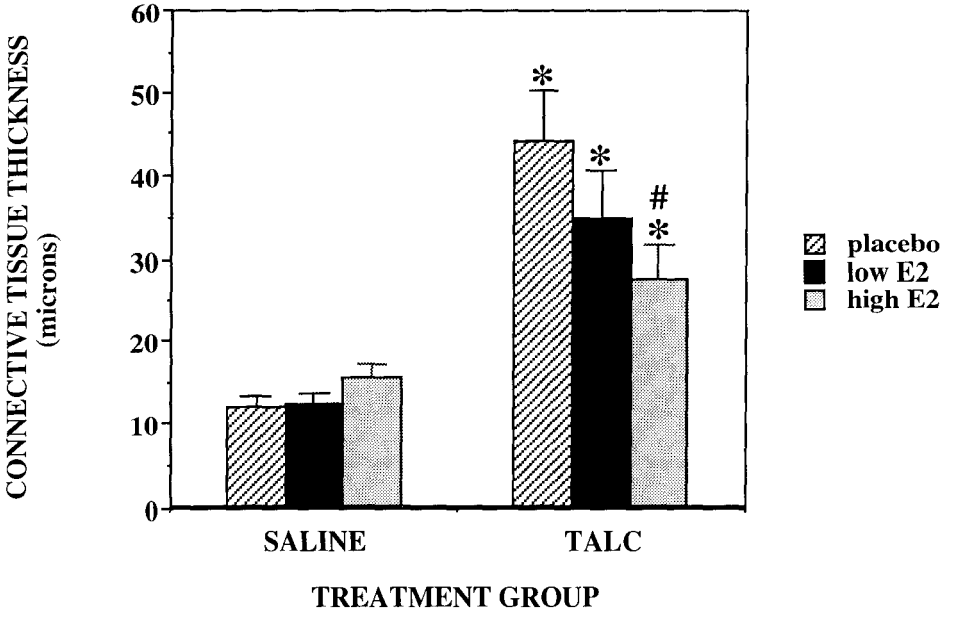

Figure 10. Effect of E_2 Replacement on Peritoneal Connective Tissue Thickness in Ovariectomized Mice Receiving Intraperitoneal Injection of Talc (30 mg) in Saline. Thickness, in microns, was assessed by light microscopy of Masson's Trichrome stained abdominal wall tissue (see Materials and Methods). Data shown are means $\pm$ SEM. *$p<0.01$ from PBS-treated controls. #$p<0.01$ from talc-treated placebo control.

Peritoneal Cell Populations

To determine whether administration of talc and/or E_2 treatment caused alterations in the resident peritoneal cell populations, absolute and differential counts of peritoneal lavage cells were performed. Attempts were made to analyze the total lavage cell numbers in all treatment groups. Because of the severity of adhesions in the talc-treated animals, quantitative recovery of peritoneal cells was difficult, as previously described (Frazier-Jessen and Kovacs 1993).

TABLE III: Resident Peritoneal Cell Populations

Treatment Group[a]	%Neutrophils[b]	%Macrophages[b]	%Lymphocytes[b]
placebo/saline	8.33 ± 3.45	69.60 ± 3.25	21.75 ± 4.99
placebo/talc	19.13 ± 3.85*	46.53 ± 3.63*	34.32 ± 6.25
low E_2/saline	9.75 ± 3.75	67.05 ± 7.85	21.95 ± 4.35
low E_2/talc	25.10 ± 5.03*	58.56 ± 6.19	16.36 ± 1.63
high E_2/saline	10.33 ± 1.33	66.03 ± 5.29	23.43 ± 4.42
high E_2/talc	20.60 ± 1.83**	53.03 ± 6.34	26.37 ± 5.48

[a]Mice were ovariectomized and implanted with pellets that released a total of 0.05 mg E_2 (low E_2), 5 mg E_2 (high E_2), or vehicle (placebo) over a 21-day period. Seven days post-ovariectomy/E_2 replacement, mice were injected i.p. with talc (30 mg in saline) or saline alone. Fourteen days later, animals were sacrificed and peritoneal lavage was performed. Resident peritoneal cell populations were differentially assessed by Wright's Modified Giemsa stain (see Materials and Methods).

[b]Data are expressed as mean + SEM.

*$p < 0.01$ between saline- and talc-treated animals of each E_2 group.
**$p < 0.03$ between saline- and talc-treated animals of each E_2 group.

It is likely that the poor and inconsistent cell recovery observed in the talc-treated mice that results, in part, from sequestration of peritoneal cells at adhesion sites.

Differential cell counts yielded the percentages of neutrophils, macrophages, and lymphocytes in peritoneal lavage fluid (Table III). Talc treatment caused a significant increase in the proportion of peritoneal neutrophils over that obtained from saline-treated animals ($p < 0.01$). No difference was observed between the peritoneal cell populations in saline-treated animals given E_2 or placebo pellets. There was a significant decrease in

the peritoneal macrophage population in talc-treated placebo animals compared with saline-treated placebo animals ($p < 0.01$), possibly resulting from adhesiveness of the cells following talc administration. This was not observed in talc-treated animals receiving either low or high E_2 replacement, suggesting that hormone treatment may decrease macrophage activation or migration in vivo. Finally, no significant differences in peritoneal lymphocyte populations were observed between any of the experimental groups.

Immunohistochemical Detection of Macrophages in Adhesion Tissue

Since macrophages are believed to play a central role in inflammation and wound repair (Leibovich and Ross 1975), we wanted to determine whether E_2 treatment affected the number of macrophages present in the connective tissue of talc-treated animals. Immunohistochemical analysis of abdominal wall tissue using the F4/80 monoclonal antibody for detection of macrophages revealed the presence of macrophages in connective tissue was observed between placebo and high E_2 treatment groups (9.9 and 8.8 cells per 100 μm^2, respectively), even though E_2 replacement decreased connective tissue deposition. The number of macrophages per unit area of adhesion tissue reported herein did not differ from that of connective tissue in models of skin wound healing (DiPietro et al. 1995; Wong et al. 1993). However, it was greater than in the connective tissue of normal undamaged murine skin (DiPietro et al. 1995) and other organs (Kovacs, unpublished observations).

Expression of JE/MCP-1 mRNA in Abdominal Wall Tissue of Talc-Treated Mice

Since E_2 replacement diminished peritoneal adhesion formation and we recently reported that E_2 suppresses expression of JE/MCP-1 mRNA in cultured murine macrophages (Frazier-Jessen and Kovacs 1995), we wanted to determine whether expression of this gene in adhesion tissue could be responsible for the macrophage recruitment to or activation at sites of tissue damage. Northern blot analysis revealed that JE/MCP-1 mRNA transcripts were not detected in abdominal wall connective tissue samples obtained from four saline-treated mice. In contrast, the message was expressed in samples from five of six talc-treated OVX mice given placebo or no E_2 replacement (Figure 11, lanes 4-7) and in only one of five samples from talc-treated OVX mice given E_2 replacemnt (three of which are shown in Figure 11, lanes 8-10). In these experiments, RNA from abdominal wall tissue from untreated animals (lane 1) and a murine macrophage cell line, ANA-1, with LPS (1 μg/ml) (lane 2) served as negative and positive controls, respectively. Table IV shows a densitometric analysis of the Northern blot. In addition, we monitored the expression of other pro-inflammatory and fibrogenic cytokines, including IL-1α, IL-1β, TNF-α, TGF-β1, and platelet-derived growth factor α- and β-chains. We observed that the

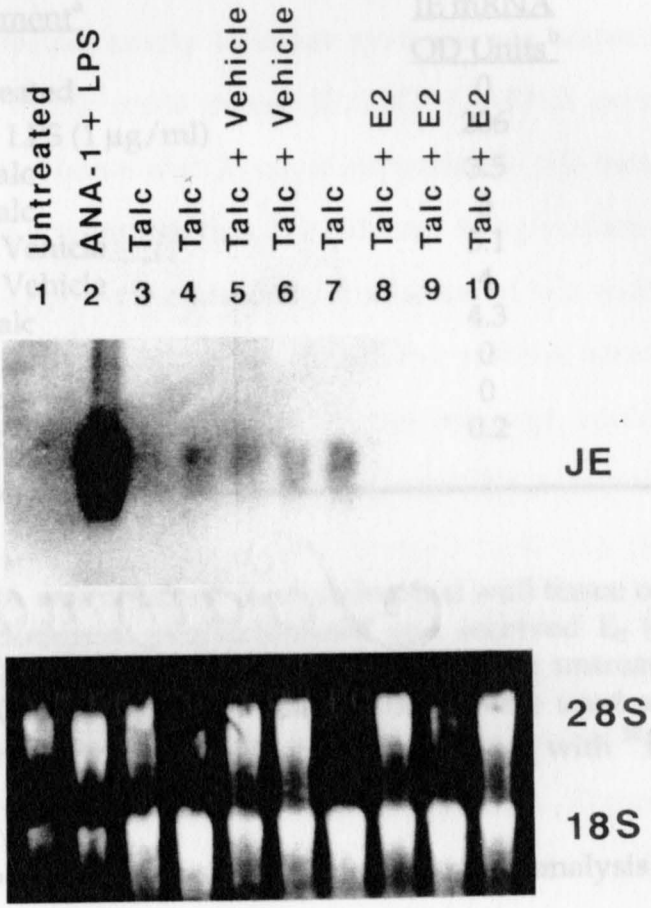

Figure 11. Expression of JE mRNA in Murine Abdominal Wall Tissue. Abdominal wall tissue from C57BL/6 mice receiving i.p. talc was homogenized and total cellular RNA extracted. Ovariectomized animals (lanes 3, 4, and 7), ovariectomized animals receiving vehicle (lanes 5 and 6) and animals receiving E_2 at 300 pg/ml (lanes 8, 9, and 10) are shown. RNA from Abdominal wall tissue of untreated animals and RNA from LPS-stimulated ANA-1 macrophages were used as negative and positive controls, respectively. Blot was hybridized with a ^{32}P-labeled JE cDNA probe. The ethidium bromide stained agarose gel is shown to demonstrate even loading of samples.

Table IV: Densitometric Scan of Northern Blot of JE mRNA Expression from Murine Abdominal Wall Tissue

Treatment[a]	JE mRNA OD Units[b]
Untreated	0
ANA-1 cells + LPS (1 μg/ml)	206
Talc	3.5
Talc	5
Talc + Vehicle	5.1
Talc + Vehicle	4
Talc	4.3
Talc + E_2	0
Talc + E_2	0
Talc + E_2	0.2

[a] Total cellular RNA was obtained from abdominal wall tissue of C57BL/6 mice that were ovariectomized, ovariectomized and received E_2 (300 pg/ml), or vehicle only. RNA from abdominal wall tissue of an untreated animal and RNA from LPS-stimulated ANA-1 macrophages were used as negative and positive controls, respectively. The blot was hybridized with ^{32}P-labeled cDNA probes for murine JE.

[b] Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

expression of these cytokine genes in abdominal wall tissue samples was below the level of detection. This may have been due, in part, to the selection of late time point examined (14 days post-talc treatment).

Estrogen Inhibition of Talc-Induced Expression of JE/MCP-1 mRNA in Murine Macrophages

Since peritoneal macrophages play a role in the generation of connective tissue in the abdominal cavity in other systems, we wanted to determine whether exposure to talc could induce JE/MCP-1 mRNA expression in vitro, and if so, whether treatment with E_2 could suppress the talc-induced expression of the message. To accomplish this, we cultured thioglycolate-elicited murine peritoneal macrophages in the presence or absence of talc with or without E_2. Minimal or undetectable levels of JE/MCP-1 mRNA were expressed in untreated macrophages. Expression of the message could be increased following 8 h of exposure to increasing concentrations of talc (from 0.01 to 100 μg/ml). At 100 μg/ml, this level ranged from 19- to 32-fold that of untreated cells, which respresents one-fifth of the maximal levels of JE/MCP-1 mRNA induced after exposure to 1μg/ml LPS. While E_2 alone had no effect on expression of the message, the addition of E_2 to talc-treated macrophages resulted in inhibition of expression of JE/MCP-1 mRNA. The concentrations of E_2 used in these studies were at or below the circulating hormone levels achieved by implantation of E_2 pellets on OVX mice. Maximal inhibition of JE/MCP-1 mRNA expression in response to talc (100 μg/ml) was observed at 300 pg/ml of E_2. In three separate experiments, the inhibition at this dose of E_2 was 94% that of cells treated with talc alone.

Since thioglycolate-elicited peritoneal macrophages purified by adherence contain small of numbers of contaminating cells (Radzioch, Bottazzi, and Varesio 1987) #123], we wanted to confirm that 1) talc treatment coulddirectly induce expression of JE/MCP-1 mRNA in the absence of other cell types, and 2) E_2 exposure could suppress expression of the message in

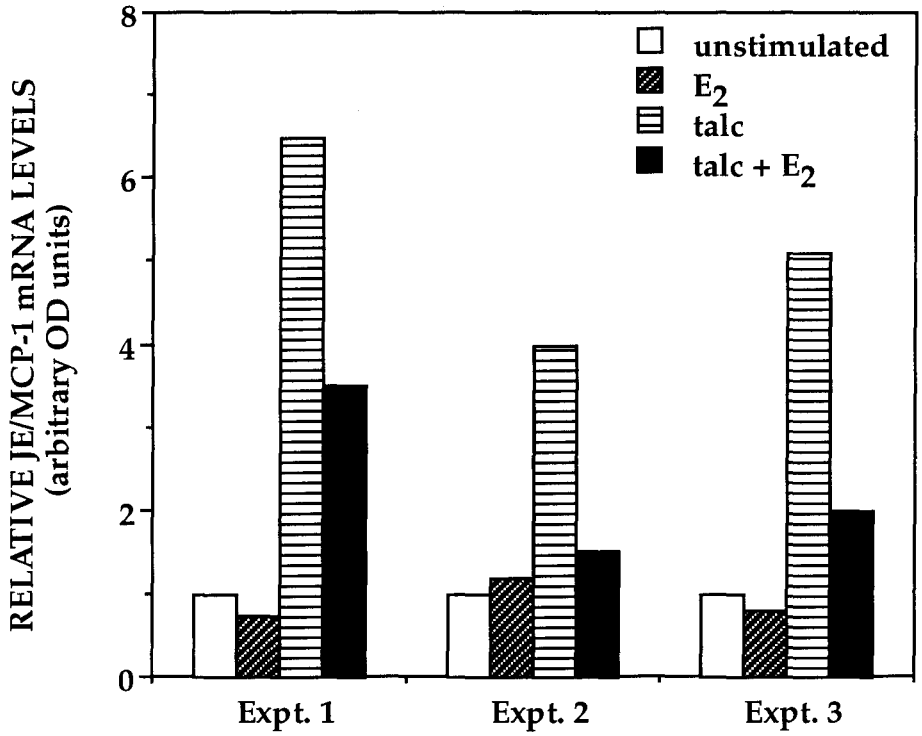

Figure 12. E_2 Inhibition of JE/MCP-1 mRNA Expression in Talc-Stimulated ANA-1 Murine Macrophages. ANA-1 cells were cultured, as described in Materials and Methods, for 8 h in the absence of stimulants, with E_2 alone (300 pg/ml), with talc alone (100 μg/ml), or a combination of talc and E_2, after which cells were harvested for RNA extraction. Quantitative analysis of E_2 inhibition of talc-induced JE/MCP-1 mRNA expression. Data are shown as the steady state level of JE/MCP-1 mRNA expression divided by the level of G3PDH mRNA expression in arbitrary OD units.

pure populations of macrophages. To accomplish this, parallel studies were performed with the ANA-1 macrophage cell line derived from a male C57BL/6 mouse. Like thioglycolate-elicited peritoneal macrophages, the ANA-1 macrophages did not spontaneously express JE/MCP-1 mRNA, but could be induced to express the message after exposure to talc for 8 h (Figure 12). While E_2 alone had no effect on JE/MCP-1 mRNA expression, the addition of E_2 to talc-treated cells resulted in a dose-dependent inhibition of expression of the

message. As with peritoneal macrophages, maximal inhibition of expression was achieved at a concentration of 300 pg/ml of E_2 and ranged from 50 to 88%. These data show that a pure macrophage population is capable of expressing JE/MCP-1 mRNA in response to talc treatment. Furthermore, since E_2 can inhibit the expression of the message in talc-treated macrophages from male mice, it suggests that both male and female mice have estrogen receptors (Frazier-Jessen and Kovacs 1995; Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986) as well as other machinery necessary to respond to estrogen treatment.

Discussion

In this study, we show that E_2 suppresses connective tissue deposition in a murine model of peritoneal adhesion formation. Our observations are consistent with those of other laboratories examining dermal wound healing in estrogen-treated ovariectomized female rats (Roth, Harman, and Lamberg 1981), and rabbits (Dyson and Joseph 1971). In these studies, daily s.c. administration of high doses of estradiol attenuated wound healing, as assessed by the time of wound closure in the rodent study (Roth, Harman, and Lamberg 1981) and regenerative growth in the rabbit study (Dyson and Joseph 1971).

The effects of E_2 on connective tissue deposition have several important physiologic implications. The loss of circulating E_2, as occurs during menopause, may permit exacerbation of inflammatory responses. This is substantiated by clinical observations of increased serum levels of several inflammatory cytokines in postmenopausal women (Pacifici et al. 1990; Ralston, Russell, and Gowen 1990). Therapeutic E_2 replacement in postmenopausal women may prevent potentially harmful inflammatory responses from

occurring. This has already been shown to be true in treatment of osteoporosis and in reducing the risk of atherosclerosis (Knopp 1988). In addition, women undergoing cesarean section show a relative paucity of postsurgical i.p. adhesion formation (Golan et al. 1991) as compared with patients undergoing other abdominal surgeries. Furthermore, decreased symptoms (i.e., pain and joint swelling) were noted in women with rheumatoid arthritis during pregnancy (Hench 1938; Vandenbroucke et al. 1986). These phenomena may be due, in part, to the presence of elevated circulating E_2 levels that are achieved during pregnancy (Carr 1990). High levels of E_2 could attenuate the local macrophage-mediated inflammatory/immune response, and thereby suppress connective tissue deposition.

Mechanism(s) by which E_2 exerts its effects on the inflammatory and fibrotic responses has not been fully addressed. Reports have demonstrated elevated expression of JE/MCP-1 mRNA in atherosclerotic vessels (Koch et al. 1993; Nelken et al. 1991; Takeya et al. 1993; Yu et al. 1992), fibrotic lungs (Brieland et al. 1992; Flory, Jones, and Warren 1993), and dermal wound healing (DiPietro et al. 1995). The correlation between the incidence of expression of JE/MCP-1 mRNA in abdominal wall tissue and the amount of connective tissue deposited following E_2 treatment suggests that hormone may diminish adhesion formation by blocking the recruitment or activation of macrophages. Taken with our data on immunohistochemical localization of macrophages in adhesion tissue, it is reasonable to suggest that the hormone may also attenuate the production of other proinflammatory and fibrogenic cytokines that can directly trigger fibroblast proliferation and collagen synthesis (Kovacs 1991; Kovacs et al. 1993). This is currently being pursued in our laboratory.

It is also possible that the effects of E_2 on peritoneal adhesion formation are mediated indirectly by estrogen stimulation of glucocorticoid production, which has been shown to decrease or delay wound healing (Leibovich and Ross 1975), and inhibit the proliferation of fibroblasts (Ponec et al. 1977; Saarni and Tammi 1978) and the synthesis of collagen (Cockayne et al. 1986; Walsh, LeLeiko, and K.M. Sterling 1987), the hallmarks of the fibrotic response. Such a mechanism is also supported by the observation that glucocorticoids suppress the expression of JE/MCP-1 mRNA (Kawahara, Deng, and Deuel 1991; Mukaida et al. 1991). However, the fact that E_2 inhibits expression of the message in talc-treated macrophages (Figure 12) demonstrates a direct means by which the hormone could alter cytokine gene expression. This is supported further by studies showing E_2 suppression of macrophage activation in rodent (Frazier-Jessen and Kovacs 1995; Sato et al. 1991) and human (Polan et al. 1989; Ralston, Russell, and Gowen 1990) systems and platelet-derived growth factor-stimulated murine fibroblasts (Kovacs et al. 1996). The presence of ER in the two main cell types involved in scar tissue formation, namely fibroblasts (Malet et al. 1991) and macrophages (Frazier-Jessen and Kovacs 1995; Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986), further suggests that E_2 could directly modulate functions of these cells.

There are several ways in which E_2 could exhibit effect on the expression of JE/MCP-1 gene. Using DNA sequence analysis, we have identified the presence of four half-palindromic ERE motifs (Kovacs, Alberta and Stiles, unpublished observation). In other studies, identical ERE half-palindromic sequences have been shown to regulate the expression of estrogen-dependent genes (Kato et al. 1992). Furthermore, the proximal promoter of the JE gene contains a silencer element that has not yet been fully characterized (Freter et al.

1992). The presence of this element suggests that, if the half-palindromic ERE motifs do not function to regulate transcription, then E_2 may inhibit transcription of the JE gene by interacting with novel sequences.

Recent reports reveal that in addition to the interaction of estrogen/ER complex with genomic ERE, the hormone can also mediate its effects by modulating intracellular second messenger pathways as well as other genomic elements. For example, at physiologic levels E_2 stimulates the accumulation of intracellular cAMP (Aronica, Kraus, and Katzenellenbogen 1994). Webb and colleagues (Webb et al. 1995) showed that estrogen/ER complexes may also act by altering the ability of the c-jun protein to interact with c-fos to form the AP-1 transcription factor that binds to AP-1 sites in the promoter of rat (Timmers et al. 1990) and murine (Kovacs and Mott, unpublished observations) JE/MCP-1 genes. This is currently being investigated in the laboratory.

In summary, we have shown that E_2 attenuates connective tissue deposition in a model of peritoneal adhesion formation, and that this inhibition may be mediated through inhibition of macrophage activation at sites of tissue damage.

CHAPTER 5

ESTROGEN MODULATION OF JE GENE EXPRESSION IN MURINE MACROPHAGES

Abstract

The chemotactic cytokine, monocyte chemoattractant protein-1 (MCP-1), and its murine homologue, JE, have been detected in atherosclerotic lesions but not in normal arteries, implicating that these pro-inflammatory cytokines may be involved in the pathogenesis of atherosclerosis. Epidemiologic studies reveal that postmenopausal women receiving estrogen replacement for treatment of osteoporosis have a greatly reduced risk of developing cardiovascular disease. Because JE/MCP-1 and estrogen play regulatory roles in the development of atherosclerotic lesions, we chose to examine the effects of estrogen treatment on JE/MCP-1 mRNA expression in macrophages. 17β-estradiol (E_2) inhibited LPS-stimulated JE/MCP-1 mRNA expression in ANA-1 and J774A.1 murine macrophage cell lines and in thioglycolate-elicited murine peritoneal macrophages. Inhibition of JE/MCP-1 mRNA ranged from 50 to 90%, with a maximal effect occurring at a concentration of 300 pg/ml E_2. Conversely, E_2 had little effect on LPS-stimulated TNF-α mRNA production. Treatment of LPS-stimulated macrophages with moxestrol, an estrogen agonist, resulted in a similar inhibition, and the addition of the estrogen antagonist, tamoxifen, reversed E_2 inhibition of LPS-induced JE/MCP-1 mRNA expression.

Immunohistochemical analysis revealed the presence of estrogen receptors in ANA-1 cells, indicating that E_2 inhibition of LPS-induced JE/MCP-1 mRNA expression in murine macrophages may be mediated through the estrogen receptor. Thus, another mechanism whereby estrogen exerts anti-atherogenic effects may be through prevention of macrophage accumulation in the atherosclerotic lesion.

Introduction

Atherosclerosis is the predominant cardiovascular disease in the United States associated with a high mortality rate. Characteristics of the pathology of this disease include the formation of fatty, fibrous plaques within the arterial wall, accompanied by an inflammatory response that ultimately results in a partial to complete occlusion of the blood vessel. The earliest recognizable lesion in the progression of this disease is the "fatty streak" consisting, in part, of an aggregation of lipid-laden macrophages (foam cells) within the intimal wall of the artery. The recruitment of these macrophages to the lesion is believed to be mediated by chemotactic cytokines (for a review, see (Ross 1993)), such as monocyte chemoattractant protein-1 (MCP-1) (Yoshimura and Leonard 1990), which is chemotactic for mononuclear phagocytes both in vitro (Rollins, Walz, and Baggiolini 1991; Yoshimura and Leonard 1990; Yoshizuka et al. 1989) and in vivo (Zachariae et al. 1990). MCP-1 and its murine homologue, JE (Yoshimura and Leonard 1990), which was identified initially as a platelet-derived growth factor-inducible gene (Cochran, Reffel, and Stiles 1983), have been detected in atherosclerotic lesions but not in normal arteries (Koch et al. 1993; Nelken et al. 1991; Takeya et al. 1993; Yu et al. 1992), suggesting that they may play a role in the pathogenesis of atherosclerosis. Because JE/MCP-1

production can influence macrophage recruitment to sites of injury and inflammation, its regulation has clinical relevance for the treatment and prevention of atherosclerosis.

One of the factors associated with an increased risk of the development of cardiovascular disease in women is the onset of menopause (Eaker and W.P.Castelli 1987). Epidemiologic studies revealed that postmenopausal women receiving estrogen replacement therapy for treatment of osteoporosis have a reduced risk of developing cardiovascular disease (Eaker and W.P.Castelli 1987; Henderson, Paganini-Hill, and Ross 1991; Knopp 1988; Stampfer et al. 1991). This is caused, in part, by the effects of estrogen on low density lipoprotein metabolism (Sacks and Walsh 1990; Schaefer et al. 1983; Walsh et al. 1991). However, these effects alone cannot completely explain the anti-atherogenic effects of estrogen.

Recent studies have shown that estrogen receptors are present in the immune and mesenchymal cell populations involved in both inflammation and atherogenesis (Sacks and Walsh 1990; Schaefer et al. 1983; Walsh et al. 1991). This suggests that estrogen may modulate the inflammatory response by directly affecting the expression of cytokine genes in immune cells. Glucocorticoids have been shown to be anti-inflammatory in numerous systems, and the synthetic glucocorticoid, dexamethasone, inhibits JE/MCP-1 mRNA expression in both fibroblasts (Kawahara, Deng, and Deuel 1991) and macrophages (Mukaida et al. 1991; Poon et al. 1991). Other investigators have shown that the production of IL-1 (Pacifici et al. 1990) and TNF-α (Ralston, Russell, and Gowen 1990) can be altered by estrogen replacement therapy. However, direct effects of estrogen on JE/MCP-1 mRNA expression have not been examined. Because both JE/MCP-1 and estrogen may play regulatory

roles in the pathogenesis of atherosclerosis, we chose to examine whether estrogen treatment affects JE/MCP-1 mRNA expression in the macrophage. Herein we show that the estrogen, E_2, can inhibit LPS-stimulated JE/MCP-1 mRNA expression in murine peritoneal macrophages and macrophage cell lines.

Materials and Methods

Reagents

17-ß-estradiol (E_2), progesterone, tamoxifen, hydrogen peroxide, and diaminobenzidine tablets were purchased from Sigma Chemical Co. (St. Louis, MO). Moxestrol was a kind gift from Rousell Uclaf (Romainville, France). Brewer's thioglycolate and LPS were obtained from DIFCO (Detroit, MI). All tissue culture reagents and random primer labeling kit were obtained from Gibco/BRL (Grand Island, NY). The cDNA probes for murine JE and murine TNF-α were obtained from the ATCC (Rockville, MD) and Chiron Corporation (Emeryville, CA), respectively. The ER-21 rabbit polyclonal antibody against estrogen receptor was a generous gift of Dr. Geoffrey Greene (University of Chicago; Chicago, IL). Normal donkey serum, biotinylated donkey anti-rabbit IgG ($F(ab')_2$ heavy and light chains), and purified rabbit gamma globulin were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The Vectastain Elite ABC immunoperoxidase kit was obtained from Vector Laboratories, Inc. (Burlingame, CA).

Isolation and Culture of Peritoneal Macrophages

Female C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) 9 to 12 weeks old were injected intraperitoneally with 1.5 ml of 10% Brewer's

thioglycolate as previously described (Radzioch, Bottazzi, and Varesio 1987). Four days later, animals were sacrificed and peritoneal lavage was performed. Peritoneal exudate cells were plated at 2 X 10^6 cells per ml of phenol red-free RPMI 1640 medium with 2% fetal bovine serum (FBS) and allowed to adhere. After 2 hours, medium containing nonadherent cells was removed and replaced with fresh medium. The remaining cells were ~95% macrophages, as determined by Giemsa stain analysis. Macrophage cultures were "rested" for 48 hours prior to stimulation in order to diminish expression of cytokine genes triggered by adherence to tissue culture plastic (Fuhlbrigge et al. 1987).

Culture of Macrophage Cell Lines

The ANA-1 murine macrophage cell line was obtained from Dr. Luigi Varesio (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD). The cell line was derived by infection of bone marrow cells from a C57BL/6 male mouse with a recombinant retrovirus containing the myc and raf proto oncogenes (Blasi et al. 1989). The J774A.1 murine macrophage cell line was derived from a female BALB/c mouse (ATCC). All cell lines were maintained in RPMI 1640 medium with 5% FBS, 100 Units/ml penicillin, 100 mg/ml streptomycin and 2mM L-glutamine. Forty-eight hours prior to stimulation, cells were plated at 5 X 10^5 cells per ml in phenol red-free RPMI 1640 medium with 2% FBS in the absence of exogenous stimulants. Cells were harvested for RNA isolation after 8 hours incubation, unless otherwise specified.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated by a single-step guanidinium thiocyanate-phenol-chloroform method (RNAzol B; Cinna Biotecx, Houston, TX) according to manufacturer's specifications. Northern blots, prepared as previously described (Kovacs, Oppenheim, and Young 1986), were hybridized with ^{32}P-labeled cDNA probes for JE and TNF-α. Quantitation of RNA signals was performed by scanning densitometry using Ambis Quantprobe Software version 3.02 (Ambis Systems, Inc.; San Diego, CA). All studies were performed three times, unless otherwise stated.

Immunohistochemistry

Cytospin preparations of ANA-1 cells were examined for the presence of estrogen receptor using the ER-21 rabbit polyclonal antibody. Cells were immediately fixed in 4% paraformaldehyde for 10 minutes, washed in PBS for 15 minutes, and successively soaked in cold methanol and cold acetone at 4°C for 3 minutes each. After rinsing in PBS, the slides were incubated with normal donkey serum in PBS for 30 minutes to reduce nonspecific staining. The slides were then incubated successively with the polyclonal rabbit anti-estrogen receptor antibody, ER-21 (2 μg/ml) or with rabbit gamma globulin (2 μg/ml) as a control antibody for 1 hour, and then with biotinylated donkey anti-rabbit IgG for 30 minutes. Endogenous peroxidase activity was blocked for by incubation for 10 minutes in 0.3% hydrogen peroxide (in PBS) prior to a 30 minute incubation with Vectastain Elite ABC immunoperoxidase kit (1:100). Each incubation was performed at room temperature in a humidified chamber and followed by three 5-minute washes in PBS. Horseradish peroxidase enzyme activity was detected by immersing the slides in 0.45 mg/ml diaminobenzidine

(DAB) for a maximum of 15 minutes in buffer containing 0.15M NaCl, 0.05M Tris, and 0.07%H_2O_2. Slides were counterstained in methyl green and observed by bright field microscopy.

Results

Expression of JE/MCP-1 mRNA in ANA-1 Cells

To determine when the peak level of JE/MCP-1 mRNA expression occurred in ANA-1 cells, cultures were stimulated with 1 μg/ml LPS for 1, 3, 8, or 18 hours, and Northern blot analysis was performed. Induction of JE/MCP-1 mRNA was detectable as early as early as 1 hour post-LPS stimulation (Figure 13, panel A). Because peak levels of the message were achieved at 8 hours after LPS stimulation (see Table V), subsequent experiments were performed at this time point, unless otherwise noted.

To determine the optimal concentration of LPS needed to induce JE/MCP-1 mRNA expression, ANA-1 cells were stimulated with 10, 1, or 0.1 μg/ml LPS, and total cellular RNA was isolated for Northern blot analysis (Figure 13, panel B). Unstimulated ANA-1 cells did not express JE/MCP-1 mRNA, whereas at all concentrations of LPS, a stronger signal was observed at a concentration of 1 μg/ml LPS. On the basis of these results, all other experiments were performed by using 1 μg/ml LPS.

Table V: Densitometric Scan of Northern Blot of LPS-Induced JE mRNA Expression in ANA-1 Murine Macrophages

Treatment[a]	JE mRNA OD Units[b]
None, 1h	0
LPS (1 μg/ml), 1h	1.1
None, 3h	0
LPS (1 μg /ml), 3h	12.5
None, 8h	0
LPS (1 μg /ml), 8h	16
None, 18h	0
LPS (1 μg /ml), 18h	8
None, 8h	0
LPS (10 μg /ml), 8h	10
LPS (1 μg /ml), 8h	9.7
LPS (0.1 μg /ml), 8h	0.8

[a]ANA-1 murine macrophage cells were cultured in the presence of LPS as described in Materials and Methods. The blot was hybridized with ^{32}P-labeled cDNA probes for murine JE.

[b]Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

E_2 Inhibition of LPS-Induced JE mRNA Expression

To examine the effects of E_2 on JE/MCP-1 mRNA expression in ANA-1 macrophages, cells were cultured in the absence of stimulants or with LPS (1 μg/ml), E_2 (3, 30, or 300 pg/ml), or in combination (Figure 14). As previously shown, unstimulated ANA-1 cells did not express JE/MCP-1 mRNA (lane 1), and LPS induced the expression of JE/MCP-1 mRNA (lane 2). Whereas E_2 alone had no effect on expression of the message (lanes 3, 5, and 7), the

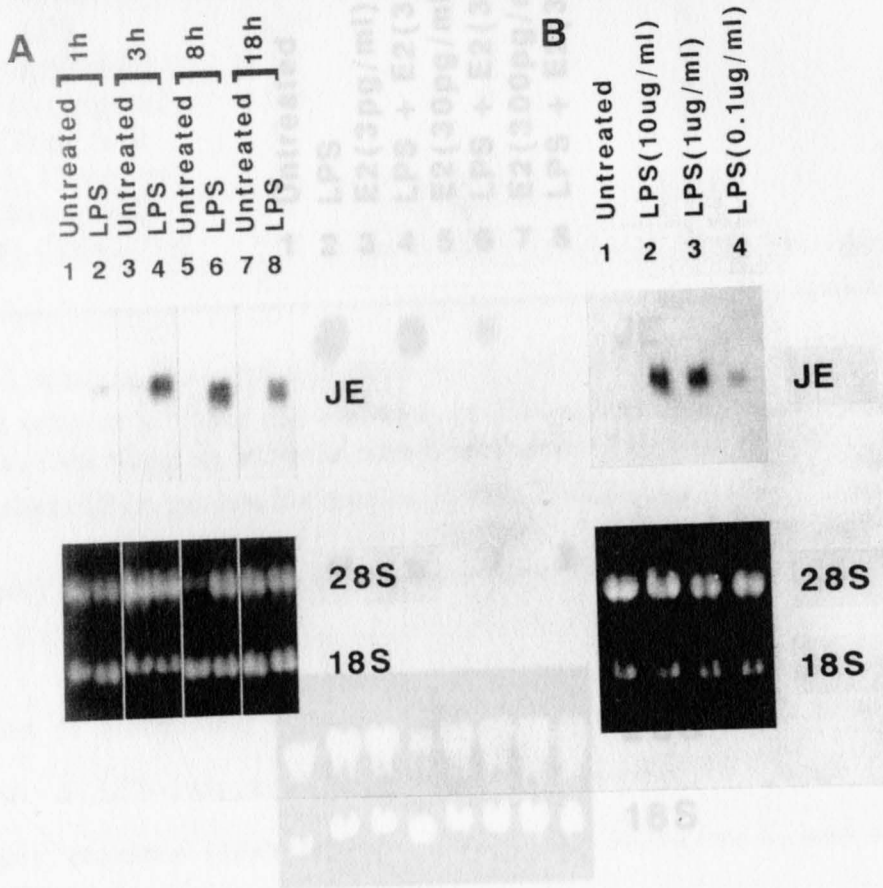

Figure 13. Expression of JE mRNA in ANA-1 Macrophages. Panel A) Untreated cells (lanes 1, 3, 5, and 7) and cells cultured with LPS (1 μg/ml) (lanes 2, 4, 6, and 8) were incubated for 1, 3, 8, and 18 hours, respectively. Total cellular RNA was extracted for Northern blot analysis. Panel B) Cells were cultured with varying doses of LPS for 8 hours after which total cellular RNA was extracted for Northern blot analysis. Untreated cells (lane 1) and cells treated with 10, 1, and 0.1 μg/ml) of LPS are shown (lanes 2-4). Representative blots were hybridized with a ^{32}P-labeled JE cDNA probe. Ethidium bromide stained agarose gels are shown to demonstrate even loading of samples.

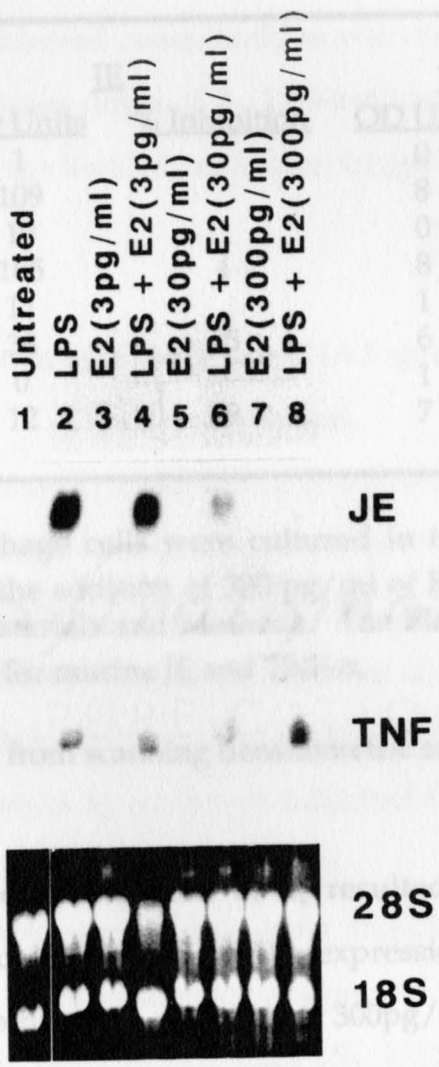

Figure 14. E_2 Inhibition of JE mRNA Expression in ANA-1 Murine Macrophages. ANA-1 cells were cultured for 8 hours with LPS (1μg/ml)) (lanes 2, 4, 6, and 8) and varying concentrations of E_2 (no E_2 - lanes 1, 2; 3 pg/ml E_2 - lanes 3, 4; 30 pg/ml E_2 - lanes 5, 6; 300 pg/ml - lanes 7, 8) after which cells were harvested for RNA extraction. A representative Northern blot was hybridized with a ^{32}P-labeled JE cDNA probe, then stripped and hybridized with a probe for murine TNF-α. The ethidium bromide stained agarose gel is shown to demonstrate even loading of samples.

Table VI: Densitometric Scan of Northern Blot of LPS-Induced JE and TNF-α mRNA Expression in the Presence or Absence of E_2

Treatment	JE		TNF-α	
	OD Units	% Inhibition	OD Units	% Inhibition
None	1		0	
LPS	109		8	
E_2 (3pg/ml)	19		0	
LPS+ E_2 (3pg/ml)	105	4	8	0
E_2 (30 pg/ml)	11		1	
LPS+ E_2 (30pg/ml)	38	65	6	25
E_2 (300pg/ml)	0		1	
LPS + E_2 (300pg/ml	12	89	7	13

[a]ANA-1 murine macrophage cells were cultured in the presence of LPS (1 μg/ml) with or without the addition of 300 pg/ml of E_2 or progesterone for 8 hours as described in Materials and Methods. The blot was hybridized with ^{32}P-labeled cDNA probes for murine JE and TNF-α.

[b]Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

addition of increasing concentrations of E_2 resulted in a dose-dependent decrease in LPS-induced JE/MCP-1 mRNA expression (lanes 4, 6, and 8). Typically, maximal inhibition was observed at 300pg/ml E_2 and ranged from approximately 50 to 90% (see Table VI). These concentrations of E_2 represent the physiologic range of circulating E_2 present in males and females during different phases of the estrus cycle and in pregnancy (Carr 1990). The maximal inhibition of JE/MCP-1 mRNA expression was specific for that cytokine, blots were stripped and reprobed for analysis of expression of another proinflammatory cytokine, TNF-α (Figure 14). Like JE/MCP-1, ANA-1 cells do not spontaneously express TNF-α, but could be induced to express the message

after stimulation with LPS. Treatment of ANA-1 macrophages with LPS and low concentrations of E_2 resulted in a slight decrease in TNF-α induction. This slight decrease was not observed consistently as was the decrease in JE/MCP-1 mRNA expression. At higher doses of E_2, LPS-induced stimulation of TNF-α was enhanced relative to the level of expression triggered in culture of ANA-1 cells with LPS alone.

E_2 Regulation of JE/MCP-1 mRNA Expression in Other Macrophages

To determine whether E_2 inhibition of JE mRNA expression was unique to the ANA-1 cell line, parallel experiments were performed using the J774A.1 macrophage cell line. Unstimulated J774A.1 cells did not express JE/MCP-1 mRNA, yet could be induced to express the message after stimulation with LPS. As was observed with ANA-1 cells, untreated cells and E_2 alone failed to affect JE mRNA expression, while E_2 treatment inhibited LPS-induced JE/MCP-1 mRNA expression of the message.

Similar observations were made using thioglycolate-elicited murine peritoneal macrophages. Peritoneal macrophages were cultured for 8 hours in the presence or absence of LPS and E_2 (Figure 15). Untreated macrophages did not express JE/MCP-1 mRNA, but could be induced to express the message following LPS treatment. Although E_2 alone had no effect on JE/MCP-1 mRNA expression, it inhibited LPS-induced JE/MCP-1 mRNA expression (see Table VII). E_2 suppression of LPS-induced JE/MCP-1 mRNA was also observed at 3 and 24 hours of culture, indicating that inhibition of JE/MCP-1 mRNA expression was not merely caused by a change in the kinetics of expression of the message.

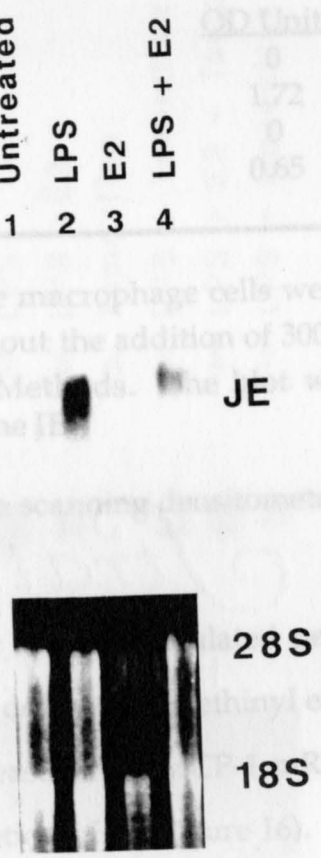

Figure 15. E_2 Regulation of Expression of JE mRNA in Thioglycolate-Elicited Peritoneal Macrophages. After incubation in the absence of stimulants for 48 hours (as described in Materials and Methods), macrophages were cultured for 8 hours in the absence of exogenous stimulants (lane 1) with LPS (1 μg/ml)) alone (lane 2) with E_2 (300 pg/ml) alone (lane 3) or with both LPS and E_2 (lane 4). A representative Northern blot was hybridized with a ^{32}P-labeled JE cDNA probe. The ethidium bromide stained agarose gel is shown to demonstrate even loading of samples.

Table VII: Densitometric Scan of Northern Blot of LPS-Induced JE mRNA Expression in the Presence or Absence of E_2

Treatment[a]	JE mRNA	
	OD Units[b]	% Change
None	0	
LPS	1.72	
E2 (300 pg/ml)	0	
LPS+E2 (300 pg/ml)	0.65	-62

[a]Thioglycolate-elicited murine macrophage cells were cultured in the presence of LPS (1 μg/ml) with or without the addition of 300 pg/ml of E_2 for 8 hours as described in Materials and Methods. The blot was hybridized with a ^{32}P-labeled cDNA probe for murine JE.

[b]Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

In addition, treatment of LPS-stimulated peritoneal macrophages with moxestrol, the 11-β-methoxy derivative of ethinyl estradiol and a highly potent estrogen, resulted in a decrease in JE/MCP-1 mRNA expression comparable with that observed with addition of E_2 (Figure 16). The specificity of moxestrol for estrogen receptor and absence of cross-reactivity with other steroid receptors (Raynaud et al. 1978) suggest that macrophages possess a high affinity estrogen binding protein and that inhibition of JE/MCP-1 mRNA expression of LPS-stimulated macrophages is mediated through this protein. To further demonstrate that the observed effects could be mediated by the classical estrogen receptor, thioglycolate-elicited murine peritoneal macrophages were treated with LPS, E_2, and the estrogen antagonist, tamoxifen, alone or in combination. The addition of tamoxifen to LPS-treated macrophages reverses

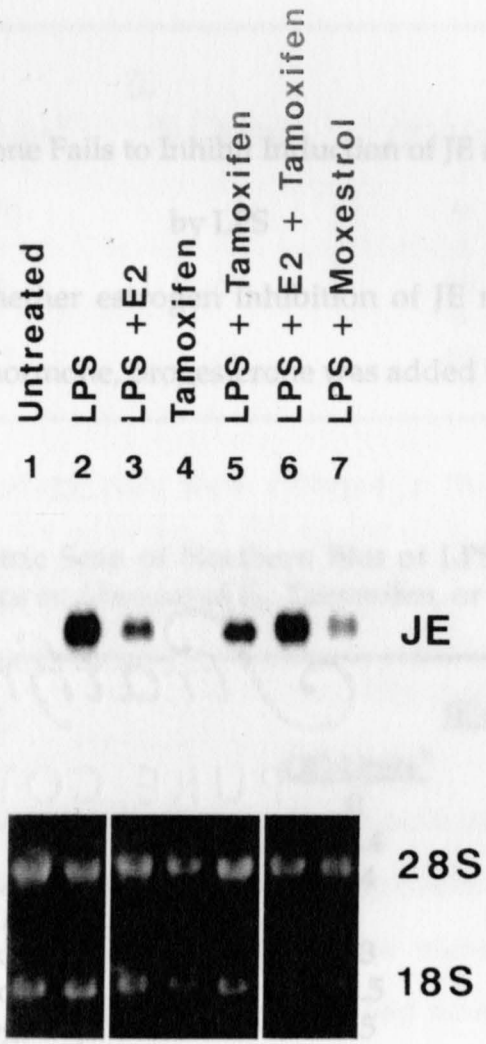

Figure 16. Moxestrol Inhibits LPS-Induced JE mRNA Expression and Tamoxifen Reverses the E_2 Inhibition of JE mRNA in Thioglycolate-Elicited Peritoneal Macrophages. Untreated macrophages (lane 1) or cells cultured for 8 hours with LPS (1 μg/ml) alone (lane 2), LPS and E_2 (300 pg/ml) (lane 3), tamoxifen (300 ng/ml) alone (lane 4), LPS and tamoxifen (lane 5), LPS, E_2 and tamoxifen (lane 6), LPS and moxestrol (300 pg/ml) are shown. A representative Northern blot was hybridized with a ^{32}P-labeled JE cDNA probe. The ethidium bromide stained agarose gel is shown to demonstrate even loading of samples.

the E_2 inhibition of JE/MCP-1 mRNA expression. Similar results were observed in ANA-1 and J774A.1 cell lines.

Progesterone Fails to Inhibit Induction of JE mRNA by LPS

To determine whether estrogen inhibition of JE mRNA expression is specific for that steroid hormone, progesterone was added to ANA-1 cultures

Table VIII: Densitometric Scan of Northern Blot of LPS-Induced JE mRNA Expression in the Presence or Absence of E_2, Tamoxifen, or Moxestrol

Treatment[a]	JE mRNA	
	OD Units[b]	% Change
None	0	
LPS	18.4	
LPS+E_2 (300 pg/ml)	6.4	-65
Tamox. (300 ng/ml)	1	
LPS+Tamox.	7.3	-60
LPS+E_2+Tamox.	18.5	
LPS+Mox. (300 pg/ml)	8.5	-65

[a]ANA-1 murine macrophage cells were cultured in the presence of LPS (1 μg/ml) with or without the addition of 300 pg/ml of E_2, 300 ng/ml Tamoxifen, or 300 pg/ml Moxestrol for 8 hours as described in Materials and Methods. The blot was hybridized with a ^{32}P-labeled cDNA probe for murine JE.

[b]Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

Table IX: Densitometric Scan of Northern Blot of LPS-Induced JE and TNF-α mRNA Expression in the Presence or Absence of E_2 or Progesterone

Treatment[a]	JE		TNF-α	
	OD Units[b]	% Change	OD Units[b]	% Change
None	1		0	
LPS	191		9	
E_2	0		1	
LPS + E_2	68	-64	19	+111
Prog	0		9	
LPS + Prog	203	+6	50	+456

[a]ANA-1 murine macrophage cells were cultured in the presence of LPS (1 μg/ml) with or without the addition of 300 pg/ml of E_2 or progesterone for 8 hours as described in Materials and Methods. The blot was hybridized with ^{32}P-labeled cDNA probes for murine JE and TNF-α.

[b]Arbitrary units obtained from scanning densitometric analysis of x-ray filters.

alone or in combination with LPS (Figure 17). In contrast to E_2, at 300 pg/ml, progesterone did not inhibit JE/MCP-1 mRNA expression induced by LPS, but rather appeared to enhance expression of the message. Furthermore, dihydroxytestosterone at the same concentration had no effect on LPS-induced JE mRNA expression. As had been previously reported by others (Kawahara, Deng, and Deuel 1991; Mukaida et al. 1991), dexamethasone inhibited LPS-induced JE mRNA expression. As shown in Figure 17, steroid regulation of TNF-α mRNA expression after steroid hormone treatment does not follow the same patterns as does JE/MCP-1. Whereas untreated cells do not express TNF-α, exposure to LPS triggers expression of the message. Exposure to 300 pg/ml E_2 fails to inhibit TNF-α mRNA expression in LPS-treated ANA-1 cells. In contrast, like JE/MCP-1, treatment of cells with a combination of progesterone

and LPS appears to enhance the level of expression of TNF-α above that of LPS alone.

Detection of Estrogen Receptor in ANA-1 Cells by Immunohistochemistry

In order to determine that the observed effects of E_2 could be mediated via the classical estrogen receptor (ER), we performed immunohistochemical analyses, using the ER-21 antibody, to insure that estrogen receptors were present in ANA-1 cells. With this antibody, cells positive for ER exhibit a strong nuclear and diffuse cytoplasmic staining. As demonstrated in Figure 18, panel A, ANA-1 cells stained positively for ER. Nearly all of the cells were positive, with some cells more strongly labeled than others. In contrast, cells incubated with a nonspecific antibody, rabbit gamma globulin (Figure 18, panel B) were negative. These observations indicate that the ANA-1 murine macrophage cell line, derived from a male C57BL/6 mouse, contains ER and is, therefore, capable of responding to estrogen treatment.

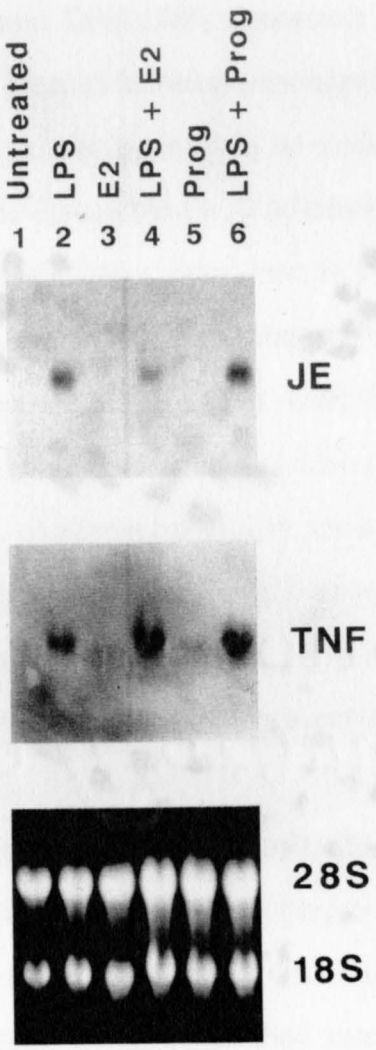

Figure 17. Expression of JE mRNA in ANA-1 Murine Macrophages. ANA-1 cells were cultured for 8 hours with LPS (1 μg/ml), E_2 (300 pg/ml) and progesterone (300 pg/ml) alone or in combination. Untreated cells (lane 1), LPS alone (lane 2), E_2 alone (lane 3), LPS and E_2 (lane 4), progesterone (lane 5) and LPS plus progesterone (lane 6) are shown. A representative Northern blot was hybridized with a ^{32}P-labeled JE cDNA probe and then stripped and hybridized with a ^{32}P-labeled TNF-α cDNA probe. The ethidium bromide stained agarose gel is shown to demonstrate even loading of samples.

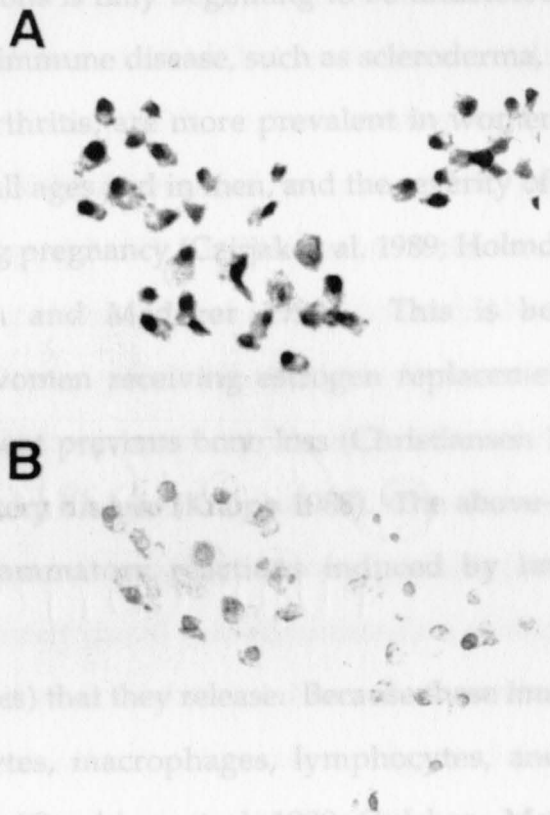

Figure 18. ER Expression in ANA-1 Macrophages. Immunohistochemistry was performed on cytospin preparations described in Materials and Methods. Cells stained with the ER-21 antibody (Panel A) or non-specific antibody, rabbit gamma globulin (Panel B).

Discussion

The sexual dimorphism of the immune response is well documented (Ansar-Ahmed, Penhale, and Talal 1985; Grossman 1985; Schuurs and Verhuel 1990), yet the role the sex steroid hormone, estrogen, plays in the regulation of immune cell functions is only beginning to be understood. Connective tissue disorders and autoimmune disease, such as scleroderma, lupus erythematosus, and rheumatoid arthritis, are more prevalent in women of reproductive age than in women of all ages and in men, and the severity of these disease states is often altered during pregnancy (Czirjak et al. 1989; Holmdahl and Jansson 1988; Steen 1990; Steen and Medsger 1990). This is best demonstrated in postmenopausal women receiving estrogen replacement therapy, in which hormone replacement prevents bone loss (Christiansen 1993) and reduces the risk of coronary artery disease (Knopp 1988). The above-mentioned conditions involve local inflammatory reactions induced by immune cells and the mediators

(including cytokines) that they release. Because these immune cell populations, including monocytes, macrophages, lymphocytes, and mast cells, express estrogen receptors (Cocchiara et al. 1990; Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986), it suggests that estrogen could directly affect cytokine production in these cells.

Chemotactic cytokines play an important role in initiating the tissue repair process by recruiting immune and mesenchymal cells into a wound site (Oppenheim et al. 1991). Therefore, modulation of these mediators can ultimately affect the outcome of the inflammatory response. Because macrophages play a central role in wound healing (Leibovich and Ross 1975), the murine macrophage chemotactic factor, JE/MCP-1, plays a critical role in

the wound healing response, as demonstrated by recent studies in which JE/MCP-1 was shown to be present in fibrotic tissues, including pulmonary fibrosis and atherosclerosis. Glucan-induced pulmonary granulomatous vasculitis in the rat was accompanied by an increase in the local expression of MCP-1 mRNA and protein, with granuloma-associated alveolar macrophages as the predominant source of MCP-1 in the later evolution of these lesions (Flory, Jones, and Warren 1993). Administration of a neutralizing antibody directed against MCP-1 resulted in decreased granuloma formation. Furthermore, increased MCP-1 mRNA expression was observed in alveolar macrophages during bleomycin-induced pulmonary fibrosis in the rat (Brieland et al. 1993). MCP-1 was released from abdominal aortic aneurysms in greater quantities than by explants from occlusive or normal aortas, and immunohistochemical analysis revealed that the macrophage was the predominant positive cell for MCP-1 in these explants (Koch et al. 1993).

We have recently found that administration of high physiologic levels of E_2 to ovariectomized mice inhibited abdominal connective tissue deposition in a model of peritoneal adhesion formation triggered by i.p. injection of talc (Frazier-Jessen et al., in press). In these studies, we observed that JE/MCP-1 mRNA levels in abdominal wall connective tissue were less in talc-treated animals receiving estrogen replacement than in those given placebo, suggesting that estrogen may modulate JE/MCP-1's role in regulatory recruitment of macrophage to sites of inflammation and wound healing.

Herein, we report that physiologic levels of E_2 can modulate expression of JE/MCP-1 mRNA in vitro. this inhibition of LPS-induced JE/MCP-1 mRNA expression occurs in primary culture peritoneal macrophages and macrophage cell lines derived from both male and female mice. Our data suggest that

because macrophages from male and female mice respond similarly to E_2 treatment, they express comparable levels of estrogen receptors, which is consistent with the work of others (Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986). Thus, the observed differences between males and females with regard to macrophage function are likely to result from differences in the circulating estrogen levels that are several thousand-fold higher in females than in males (Raynaud et al. 1978).

The fact that tamoxifen antagonizes the E_2-dependent inhibition of JE/MCP-1 mRNA expression further suggests that the negative regulation of expression of the message by estrogen is specific for that steroid hormone. Because progesterone fails to block and, in fact, enhances LPS-induced JE/MCP-1 mRNA expression, the effects of E_2 on the expression of the JE/MCP-1 gene are unique to estrogen. This observation is in accordance with the observations of Kawahara and colleagues, demonstrating that progesterone is unable to inhibit JE/MCP-1 mRNA expression in murine 3T3 fibroblasts (Kawahara, Deng, and Deuel 1991). This is not surprising, as progesterone often acts in opposition to estrogen.

Only a handful of studies have reported on the effects of gonadal steroids on the expression of cytokine genes in purified immune cell populations. Hu and coworkers showed that adherent peritoneal cells obtained from intact female rats secreted greater amounts of IL-1 than cells from male or ovariectomized female rats (Hu, Mitcho, and Rath 1988). The increased production of IL-1 was restored after E_2 replacement in OVX animals. IL-1β mRNA expression in LPS-stimulated human monocytes was elevated marginally in response to low physiologic levels of E_2 and inhibited markedly after treatment with high doses of E_2 (Polan et al. 1989). The doses required to

diminish LPS-induced expression of IL-1β mRNA were 10^{-7} to 10^{-9} M E_2, well above the physiologic range. The requirement for such high doses suggests that E_2 may not be utilizing specific high affinity receptors, but rather low affinity estrogen binding sites reported to be present on peripheral blood mononuclear cells (Ranelletti et al. 1988; Wada et al. 1992). At physiologic levels, E_2 had marked inhibitory effects on the production of TNF-α by human peripheral blood mononuclear cells from postmenopausal women. This difference may be caused by the relative level of estrogen receptors in cells from pre- and postmenopausal women. Our studies show that JE/MCP-1 mRNA in LPS-stimulated murine macrophages is inhibited by E_2 in a dose-dependent manner over a physiologic range of concentrations (10^{-11} to 10^{-9} M). In contrast, TNF-α mRNA expression is not and, in fact, is enhanced by LPS-treated cells exposed to high physiologic concentrations of E_2 (10^{-9} M). Taken together with our observations, it should be clear that 1) cytokine mRNA expression can be modulated by exposure of cells to estrogen and 2) not all cytokines are coordinately regulated by the hormone. Differences between the studies of others, cited above, and our work may be attributed to differences in the species, the purity and state of activation of cell populations, and culture conditions used in the studies.

Our studies do not address the mechanism(s) by which E_2 regulates JE/MCP-1 mRNA expression, because our immunoghistochemical anlaysis show that the macrophages used in these studies have the classical estrogen receptor and suggests that E_2 suppression of expression of JE/MCP-1 mRNA might be a result of E_2-ER complexes binding to genomic estrogen response elements (ERE), as has been reported in other systems. Although regions homologous to the consensus sequence of the ERE have not been reported in

the 5′ flanking regions of the murine JE gene or the human MCP-1 genes, the proximal promoter of the JE gene contains a silencer element that has not yet been fully characterized (Freter et al. 1992). The presence of this element suggests that, if ERE sequences are not found in the promoter, then E_2 may inhibit transcription of the JE gene by interacting with novel sequences.

It is likely that future studies will confirm that E_2 inhibition of LPS induction of JE/MCP-1 mRNA in macrophages is mediated by classical high affinity estrogen receptors, because the concentrations of E_2 used in these studies are well within the range that utilizes this receptor. Alternatively, it is also possible that other pathways of estrogen regulation of gene expression are involved. For example, inositolphospholipid metabolism has been observed in E_2-stimulated human endometrial fibrobalsts by a mechanism that does not involve the classical estrogen receptor (Iida, Imai, and Tamaya 1989). Other studies show that expression of JE/MCP-1 mRNA in macrophages is induced by a combination of PMA, which activates protein kinase C, and calcium ionophore A23187, which increases cytoplasmic Ca^{2+} levels (Introna et al. 1987). Because LPS is known to mediate its effects on cytokine gene expression in macrophages by triggering protein kinase C and calmodulin-dependent kinase (Kovacs et al. 1989; Kovacs et al. 1988), the modulatory effects of E_2 on these second messenger pathways could provide an additional mechanism by which E_2 could inhibit JE/MCP-1 mRNA expression.

In summary, our data demonstrate that at high physiologic levels, E_2 inhibits LPS-induced JE/MCP-1 mRNA, and suggest that the local migration of macropahges to wound sites may be impaired by E_2. Interestingly, this observations corresponds with sex steroid levels and the associated risk of developing coronary artery disease. Estrogens protect against atherosclerosis in

a wide variety of animal models, as well as in humans (Adams et al. 1991; Barrett-Connor 1992; Knopp 1988; Stampfer et al. 1991). Even though estrogens are able to induce changes of plasma lipid and lipoprotein levels (Sacks and Walsh 1990; Schaefer et al. 1983; Walsh et al. 1991), this in itself does not fully account for the prevention of atherosclerosis. On the basis of our results and the observations of others, we propose that one possible mechanism for this protective effect of estrogen involves its ability to inhibit JE/MCP-1 mRNA expression by macrophages localized within the atherosclerotic plaque, ultimately preventing the accumulation of more macrophages, the precursors of the foam cells present within atherosclerotic lesions.

DISCUSSION

Peritoneal fibrosis and adhesion formation is the consequence of overproduction of connective tissue within the peritoneal cavity. Currently, the only therapy available for treatment of this potentially life-threatening condition is surgical intervention to lyse adhesions. While several models of adhesion formation exist (Diamond et al. 1991; Linsky et al. 1987; Myllarniemi et al. 1966), the methods of assessment are crude at best and involve a large amount of subjective interpretation.

One purpose for the studies presented herein was to develop a model of peritoneal adhesion formation that allowed for more quantitative assessment of the fibrotic response. Such a system would allow for testing of potential therapeutic agents within the peritoneal cavity. A successful therapeutic regimen would decrease connective tissue deposition and may diminish the need for further sugical lysing of adhesions.

The model developed in this dissertation involved the i.p. administration of talc (hydrous magnesium silicate), once a common contaminant of surgical gloves (Henderson et al. 1978; Kus et al. 1979). Other models of peritoneal fibrosis have involved the use of assorted particulates, such as (Renvall, Lehto, and Penttinen 1987) which is not cleared by the phagocytic cells of the immune system, resulting in a chronic inflammatory stimulus. Surgical abrasion is another commons means of eliciting chronic peritonitis (Diamond et al. 1991; Linsky et al. 1987). Herein, talc was used to allow for the administration of

different doses and, thus, the establishment of a dose-response relationship. The results obtained form the first study (see Chapter 3) showed that i.p. administration of talc caused a dose-dependent increase in connective tissue deposition on the abdominal wall. From this curve we selected a talc dose which generated detectable but not maximal adhesion thickness. Using this mid range dose of talc alllowed us to detect both upward or downward modulations following the addition of potentially therapeutic agents. In addition, the rank scoring analysis that had been previously reported in the literature was performed (Myllarniemi et al. 1966). While this analysis gave a crude measurement of the severity of adhesion formation, it did not allow for detection of subtle differences between treatment groups, thus limiting its usefulness as a critical diagnostic tool.

Estrogen is a gonadal steroid that has many as yet undescribed actions upon the inflammatory response and connective tissue composition. Replacement of estrogen in postmenopausal women remains an effective treatment for the prevention of both osteoporosis and coronary artery disease (Christiansen 1993) but little is known about the exact role of estrogen in normal connective tissue homeostasis as it appears to function through poorly understood mechanisms. Current animals models of human disease frequently use male subjects in order to eliminate any confusion caused by cycling female hormones (Dresser 1992). However, the usefullness of observations derived from these studies is questionable, as physicians must prescribe treatment for women of child-bearing age as well as men and postmenopausal women. Furthermore, as recent epidemiological studies have shown that menstrual cycle status at the time of mastectomy correlates with breast cancer recurrence (Bluming and Hrushesky 1991), an understanding of the potential modulatory

affects of gonadal hormones on biological functions may be critical to the success of the prescribed therapy.

Because of the potentially important effects of estrogen on inflammation and wound healing, it was hypothesized that estrogen influenced the inflammatory response and ultimately connective tissue compostion. We chose to examine estrogen's potential effects in our model of talc-induced peritoneal adhesion formation in the next set of experiments. The data from this second study demonstrated that E_2 administration to ovariectomized mice resulted in inhibition of connective tissue deposition in the peritoneal cavity. The doses administered were designed to deliver both low (30 pg/ml) and high (3000 pg/ml) circulatory levels of E_2, representing serum proestrus levels and serum levels comparable to the latter stages of pregnancy, respectively. Connective tissue deposition in the peritoneal cavity was dose-dependently inhibited in response to estrogen treatment. Interestingly, when the resultant connective tissue thicknesses obtained from the first study (Figure 7) are compared with those obtained from the second study (Figure 10), talc-treated (30 mg), nonovariectomized mice had a mean connective tissue thickness that was slightly lower than talc-treated ovariectomized mice. This suggests that removal of the ovaries, the major site of E_2 synthesis, results in a hyperresponsiveness to talc by way of increased connective tissue deposition. E_2 is thought to mediate its actions by binding to intracellular receptors in target cells and ultimately altering gene transcription. Clearly, a dramatic alteration in circulating E_2 could result in a different profile of such gene products, that are either directly or indirectly are involved in inflammation and connective tissue remodeling. It has been known for many years that gonadectomy not only increases body weight (Geiselman and Almli 1978), but eventually leads to

thymic and splenic hypertrophy (Nandedkar 1992; Utsuyama and Hirokawa 1989). B and T lymphocyte populations increase in both spleen and bone marrow as well as B lymphocyte precursors (Masuzawa et al. 1994; Utsuyama and Hirokawa 1989). These observations suggest that E_2 and other sex steroids somehow control lymphocyte proliferation/expansion and activation. In response to E_2 loss, an increase in both sheer number and activational status of peripheral immune cell populations could conceivably shift the response to an inflammatory stimulus to a more pathological outcome. Recent studies have demonstrated that E_2 levels in women are substantially reduced following menopause and/or surgical removal of ovaries which directly correlates with an increased risk for development of both osteoporosis and coronary artery disease. Concurrent with this is an observed increase in serum IL-1 and TNF-α levels of these women (Pacifici et al. 1990; Ralston, Russell, and Gowen 1990). Both IL-1 and TNF-α are intimately involved in connective tissue remodeling in the bone microenvironment and the vascular system and excessive levels have been associated with pathological changes. E_2 replacement by itself returns serum IL-1 and TNF-α back to baseline circulating levels, while progesterone replacement is ineffective. Thus our results and the observations of others demonstrate an important role for E_2 in normal immune function and homeostasis.

Our in vivo studies did not address the potential immunomodulatory effects of steroidal hormones besides E_2 that are synthesized within the ovary. We chose to focus on E_2, as it is the major steroidal product of the ovary. Other potentially relevant hormones include progesterone, testosterone (a precursor in estrogen synthesis), and dihydroepiandrostenedione (DHEA). In the reproductive tissues, progesterone typically has opposing activities in contrast

to estrogen. Also, as stated earlier, progesterone replacement alone does not improve cardiovascular outcome nor risk of osteoporosis in women at menopause nor after surgical ovariectomy (Christiansen 1993). Progesterone is also not able to reduce increased circulating TNF-α/IL-1 levels in these women (Pacifici et al. 1990; Ralston, Russell, and Gowen 1990). DHEA levels decrease as individuals age and several recent studies have shown a direct correlation of immune function in aged versus young animals (Weksler 1993). Thus, our results cannot discount that these other steroids may play a role in connective tissue remodeling.

In addition, the cells in the peritoneal cavity may not be involved in the deposition of connective tissue to the same extent as cells that have already migrated to the wound site. The latter population of cells can directly influence the local wound site by secreting cytokines which influence the production of extracellular matrix molecules by fibroblasts. In order to address this issue, we performed immunohistochemical analyses on abdominal wall tissue using the F4/80 monoclonal antibody, a specific marker for cells of the macrophage lineage (Hume, Loutit, and Gordon 1984; Hume et al. 1983). While these experiments revealed that macrophages were present in high numbers in adhesion tissue, the number of macrophages per area of adhesion/connective tissue did not differ between talc-treated animals receiving estrogen versus those that did not receive estrogen, even though the amount of connective tissue deposited in these animals did differ. We further demonstrated the expression of the monocyte chemoattractant protein, JE, in abdominal tissues taken from talc-treated mice and that JE mRNA was not expressed in talc-treated mice receiving E_2 replacement, suggesting a mechanism for our observations. Since macrophages are present in the adhesion tissue of our

peritoneal model, it is likely that they play an active role in the development of these adhesions, confirming previous observations that these cells are key players in the resolution of wound healing (Leibovich and Ross 1975).

To monitor peritoneal adhesion formation, animals were sacrificed on day 14 post-talc injection, since preliminary histological studies revealed that fibrin present in adhesion tissue in response to i.p. talc administration was not converted to collagen, as detected by trichrome staining, (MR Frazier-Jessen, unpublished observation) until day 14. To begin to examine mechanisms which could explain extrogen mediated effects on connective tissue deposition, we monitored peritoneal cell populations. This analysis at day 14 did not reveal any differences between talc-treated and saline controls animals. While these results were disappointing in view of the differences in connective tissue accumulation in the treatment groups, there are several factors that could explain our observations. One such possibility involves the kinetics of cellular migration in response to an inflammatory stimulus which follows essentially the same pattern irregardless of the tissue/organ. Typically, there is an immediate recruitment of inflammatory cells to the site of injury. The first cells to arrive at the site consist mainly of neutrophils. If the neutrophil population is unable to clear the irritant, macrophages are elicited to the site of inflammation. In instances of chronic inflammation, lymphocytes are eventually recruited into the tissue site. However, while observation about changes in leukocyte populations might be better addressed at an earlier timepoint in this model, assessment of connective tissue deposition could not be determined at such an early timepoint. In addition, randomly cycling female mice were used in this first study. As demonstrated by our subsequent studies, sex steroids can greatly influence inflammatory outcomes. While not performed, a comparison

of estrus cycle status with leukocyte populations might have yielded more significant differences. Finally, one of the unforeseen complications resulting from i.p. talc injection was that analysis of total cell numbers present within the peritoneal cavity was difficult to monitor, because adhesion prevented collection of cells, especially following administration of higher doses of talc. Accurate information would have been useful in gaging the magnitude of inflammation elicited and/or total increases in leukocyte populations.

In the final study, an examination of a possible mechanism by which E_2 might be inhibiting connective tissue deposition was performed. As previously discussed, macrophages are critical to the wound healing response and they have been linked to the development of peritoneal adhesions and fibrosis in numerous abdominal pathologies (Kovacs 1991; Leibovich and Ross 1975; Simons et al. 1992). Therefore, it was hypothesized that recruitment of these macrophages is a key factor in the outcome of the inflammatory response and studies were conducted to determine whether E_2 could modulate JE (murine homologue of MCP-1) mRNA expression in murine macrophages in vitro. This investigation revealed that E_2 inhibited JE mRNA expression triggered by LPS or talc stimulation in several macrophage cell lines and in thioglycolate-elicited murine peritoneal macrophages. This effect was dose-dependent, with maximal inhibition occurring at 300 pg/ml. Furthermore, this inhibition was specific for estrogen in that progesterone and DHT did not inhibit JE mRNA expression. An estrogen agonist, moxestrol, also inhibited JE mRNA expression and an estrogen antagonist, tamoxifen, was able to reverse this inhibition. Immunohistochemical analysis revealed that the classical ER are present in macrophages, confirming the work of others (Gulshan, McCruden, and Stimson 1990; Weusten et al. 1986). Since the concentrations of estrogen used in these

studies are well within the range utilized by this receptor, it suggests that the observed phenomena were mediated through the classical estrogen receptor.

The exact mechanism by which E_2 inhibits JE mRNA expression is not known and was not addressed in this dissertation. While regions homologous to the consensus sequence of the estrogen response elements (ERE) have not been reported in the JE promoter, several "ERE-like" elements have been identified in the 5' flanking regions of murine, rat, and human JE/MCP-1 genes (A. Weisz, unpublished observation). In addition, the proximal promoter of the JE gene contains a silencer element, which has yet to be characterized (C.D. Stiles, personal communication), suggesting that, if functioning ERE sequences are not found, E_2 may inhibit transcription of the JE gene by interacting with novel sequences in the silencer element.

Alternatively, it is possible that E_2 suppression of JE mRNA expression involves blocking the second messenger pathways that lead to induction of expression of the message rather than activating an antagonistic pathway. Elevation of intracellular cyclic AMP (cAMP) has been shown to suppress LPS-induced JE mRNA expression in macrophages (Tannenbaum and Hamilton 1989) and, therefore, it is possible that E_2 inhibition of JE mRNA expression results from elevation of cAMP levels. Finally, E_2/ER complexes may interfere with the binding of cis-acting proteins to genomic elements that are known to be present in the 5' flanking region of the JE gene. The ERE of the human c-fos gene binds the E_2/ER complex and the AP-1 transcription factor (Weisz and Rosales 1990), suggesting that E_2 regulation of the JE gene may be accomplished by a similar mechanism. This idea is further supported by the observation that the rat JE promoter contains an AP-1 binding site (Timmers et al. 1990). Future

experiments directed at these possibilities will help to determine the mechanism of inhibition of JE gene expression by E_2.

SUMMARY

In conclusion, the studies presented in this dissertation show that estrogen inhibits connective tissue deposition in a murine model of peritoneal adhesion formation and one possible mechanism that was examined in this dissertation is the inhibition of monocyte/macrophage chemotaxis via the inhibition of JE mRNA expression by peritoneal macrophages (see Figures 19 and 20). Since estrogen appears to have inhibitory effects connective tissue deposition, the results obtained from these studies have clinical relevance in the treatment of patients with fibrotic disorders, such as peritoneal fibrosis and atherosclerosis. Conversely, if inhibition of estrogen proves to enhance wound healing, then patients with poor healing characteristics, such as individuals with cutaneous ulcers, could be given antiestrogens in order to maximize their wound healing response. Finally, these studies further emphasize that it is important for clinicians to take into consideration a patient's hormonal status when considering treatment regimens.

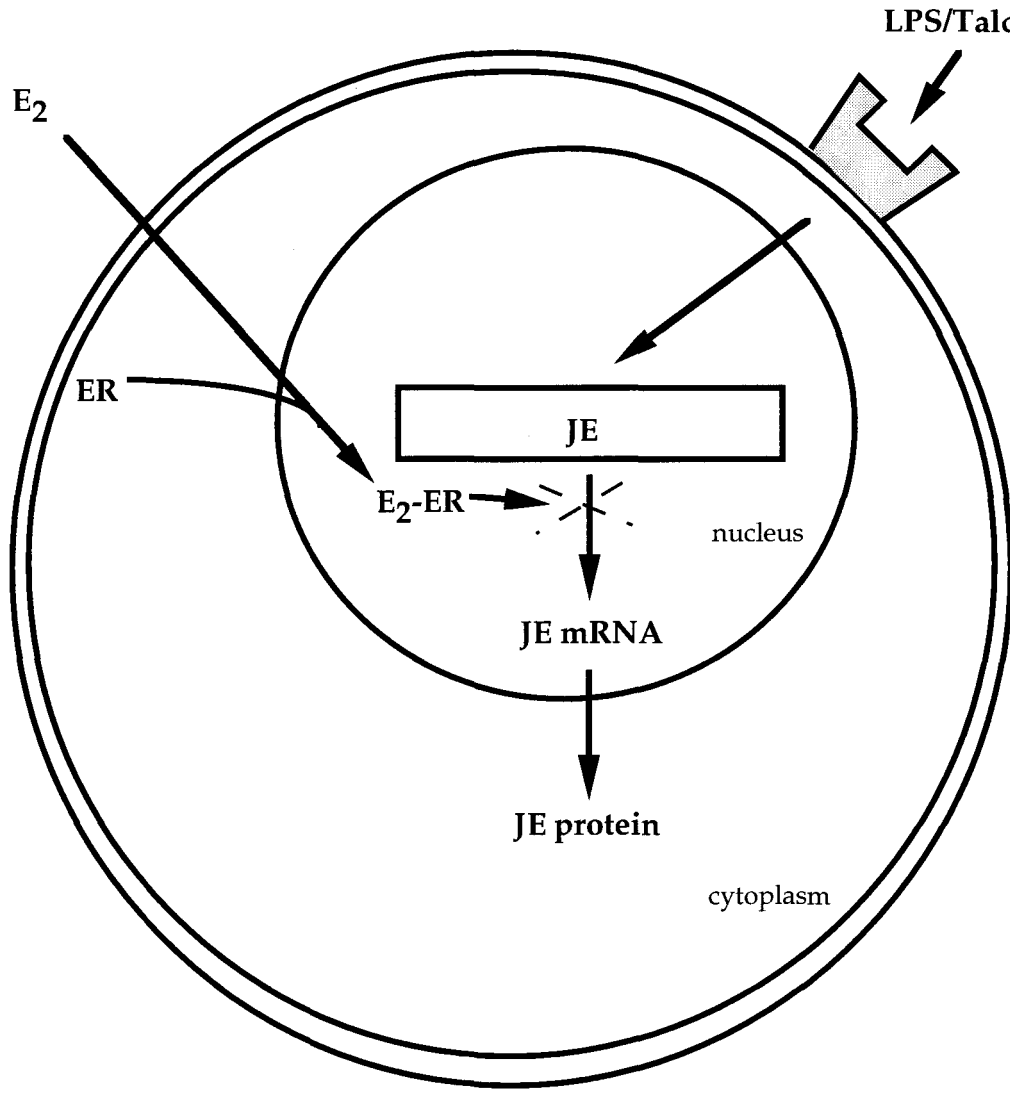

Figure 19. Proposed Effect of E_2 on JE mRNA Synthesis in Macrophages. In response to an inflammatory stimulus (such as talc or LPS) JE gene expression is upregulated, leading to synthesis of JE protein. In the presence of elevated E_2 levels, the expression of the JE gene is inhibited.

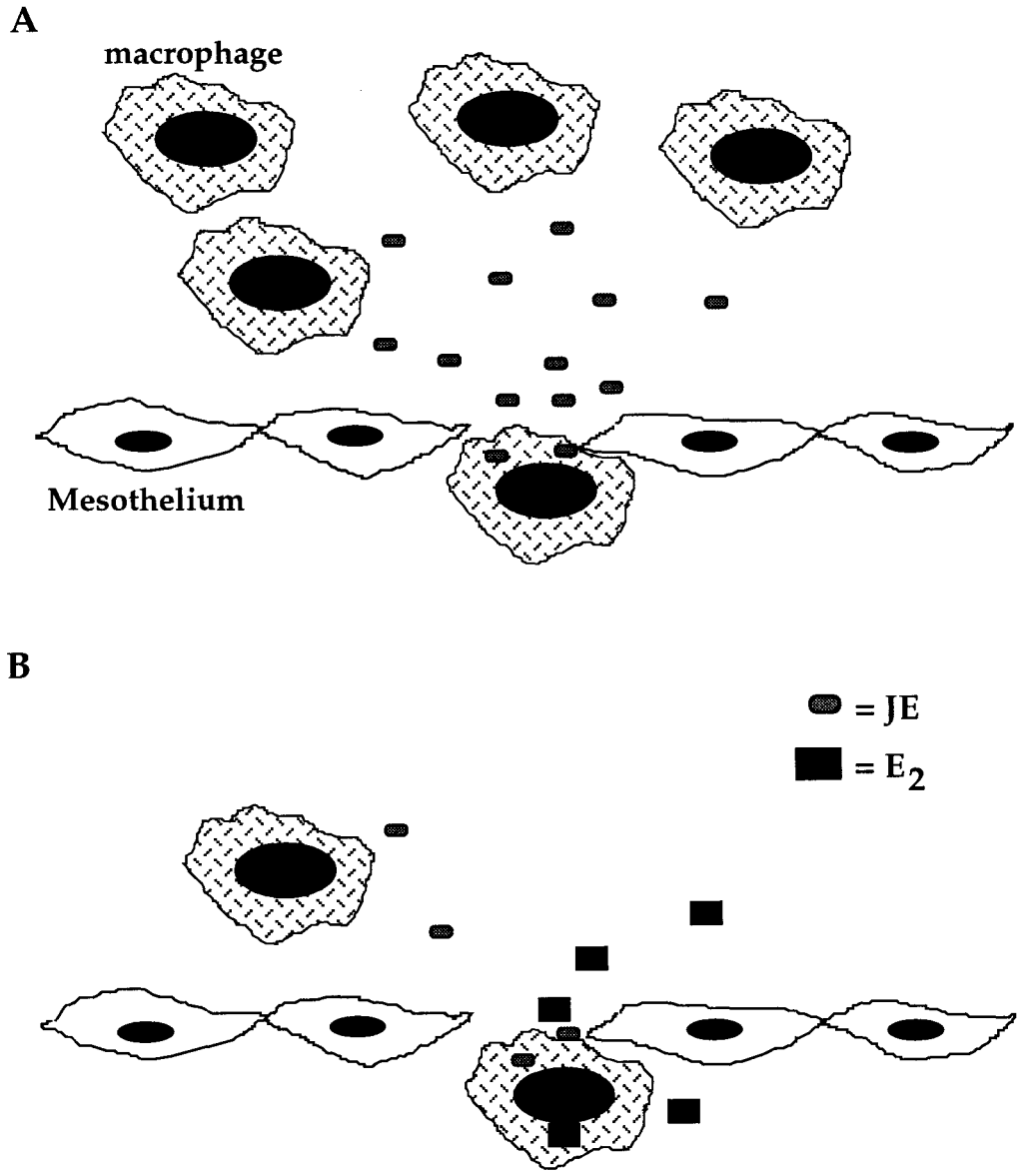

Figure 20. Proposed Effect of E_2 on Monocyte/Macrophage Chemotaxis in the Peritoneal Cavity. (A) In response to an inflammatory stimulus, monocytes are recruited via the release of chemotactic factors such as JE. (B) In the presence of elevated E_2 levels, secretion of JE is decreased, resulting in a decreased recruitment of monocytes/macrophages to the site of inflammation.

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VITA

The author, Michelle Rene' Frazier-Jessen was born on December 13, 1964 in Portland, Oregon to Dr. Marvin Frazier and Carmen Frazier.

In August, 1983, Ms. Frazier-Jessen entered Washington State University, receiving the degree of Bachelor of Science in Microbiology in June, 1988. While attending WSU, Ms. Frazier-Jessen performed independent laboratory studies for Dr. Louis P. Mallavia and Dr. James O. Schenk. During this time, Ms. Frazier-Jessen was awarded a NORCUS summer fellowship at Battelle Pacific Northwest Laboratories. As a member of the Alpha Omicron Pi Sorority, Ms. Frazier-Jessen served as Philanthropy Chairman and was also awarded the Ruby Scholarship.

In August, 1988, Ms. Frazier-Jessen was granted an assistantship in the Department of Cell Biology, Neurobiology, and Anatomy at Loyola University Chicago, enabling her to complete the Doctor of Philosophy in 1994. While at Loyola, Ms. Frazier-Jessen served as Vice President of Graduate Student Council and as a Graduate Student Representative for the Committee on Student Life.

Ms. Frazier-Jessen has accepted a postdoctoral fellowship in the laboratory of Dr. Sharon Wahl at the National Institute of Dental Research, National Institutes of Health, in Bethesda, Maryland.

APPROVAL SHEET

The dissertation submitted by Michelle R. Frazier-Jessen has been read and approved by the following committee:

Dr. Elizabeth J. Kovacs, Director
Assistant Professor, Cell Biology, Neurobiology & Anatomy
Loyola University Chicago

Dr. Linda A. Piccinini
Associate Professor, Medicine
Loyola University Chicago

Dr. Phong T. Le
Associate Professor, Cell Biology, Neurobiology & Anatomy
Loyola University Chicago

Dr. Luisa DiPietro
Associate Professor, Shock Trauma Institute
Loyola University Chicago

Dr. James P. Filkins
Professor, Physiology
Loyola University Chicago

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

4/1/96
Date

Director's Signature